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Investigation of vitamin D metabolism in non-skeletal disorders of companion animals

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Masters by Research

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Student Declaration

I declare that the thesis has been composed by myself and that the work has not be submitted for any other degree or professional qualification. I confirm that the work submitted is my own, except where work which has formed part of jointly-authored publications has been included. My contribution and those of the other authors to this work have been explicitly indicated below. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

The work presented in Chapter 2 was previously published in *PLOS as Vitamin D Status Predicts 30 Day Mortality in Hospitalised Cats* by **Helen Titmarsh**, Scott Kilpatrick, Jennifer Sinclair, Alisdair Boag, Elizabeth F. Bode, Stephanie M. Lalor, Donna Gaylor, Jacqueline Berry, Nicholas X. Bommer, Danielle Gunn-Moore, Nikki Reed, Ian Handel, and Richard J. Mellanby. Richard Mellanby also acted as supervisor for this Master Thesis. This study was conceived by Richard Mellanby, Ian Handel with input from Helen Titmarsh. I carried out experimental work, data analysis and writing of the final paper. All authors were involved in experimental work/data collection and approved the material which was also included in the paper (which differs from this chapter). Advice for the regression analysis was provided by Ian Handel.

Since initially submission of the thesis the work in Chapter 3 has published in the *Journal of Veterinary Internal Medicine as Association of Vitamin D Status and Clinical Outcome in Dogs with a Chronic Enteropathy* by **Helen Titmarsh**, Adam Gow, Scott Kilpatrick, Jennifer Sinclair, Tracy Hill, Elspeth Milne, Adrian Philbey, Jacqueline Berry, Ian Handel, and Richard J Mellanby. Richard Mellanby also acted as supervisor for this Master Thesis. This study was conceived by Helen Titmarsh and Richard Mellanby. I carried out experimental work, data analysis and writing of the final paper. All authors were involved in experimental work/data collection and approved the material which was also included in the paper (which differs from this chapter). Advice for the regression analysis was provided by Ian Handel.

Since initially submission of the thesis, the work presented in Chapter 4 has published in *PLOS* as *Low vitamin D status is associated with systemic and gastrointestinal inflammation in dogs with a chronic enteropathy* by **Helen Titmarsh**, Adam Gow, Scott Kilpatrick, Jennifer Cartwright, Elspeth Milne, Adrian Philbey, Jacqueline Berry, Ian Handel, and Richard J Mellanby. Richard Mellanby also acted as supervisor for this Master Thesis. This study was conceived by Helen Titmarsh and Richard Mellanby. I carried out experimental work, data analysis and writing of the final paper. All authors were involved in experimental work/data collection and approved the material which was also included in the paper (which differs from this chapter). Advice for regression analysis was provided by Ian Handel.

The work presented in Chapter 5 has been submitted for publication is currently under review in the *Journal of Veterinary Medicine and Science* as *Vitamin D status in cats infected with Feline Immunodeficiency Virus* by **Helen. Titmarsh**, Stephanie. Lalor, Severine Taker, Emi .N. Barker, Jacqueline Berry, Danielle Gunn-Moore, and Richard J Mellanby. Richard Mellanby also acted as supervisor for this Master Thesis. This study was conceived by Helen Titmarsh and Richard Mellanby. I carried out experimental work, data analysis and writing of the final paper. All authors were involved in experimental work/data collection and approved the material which was also included in the submitted paper.

Helen F Titmarsh

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Vitamin D Status Predicts 30 Day Mortality in Hospitalised Cats

Vitamin D status is predictive of clinical outcome in dogs with a chronic enteropathy

Low vitamin D status is associated with systemic and gastrointestinal inflammation in dogs with a chronic enteropathy

ABSTRACT

Vitamin D is traditionally known for its role in calcium homeostasis and bone metabolism. However, it has been demonstrated that numerous types of cells express the vitamin D receptor and it is now clear that the physiological roles of vitamin D extend beyond the maintenance of skeletal health. Vitamin D insufficiency, which is typically assessed by measuring the major circulating form of vitamin D, 25 hydroxyvitamin D (25(OH)D), has been associated with a number of disorders in people including hypertension, diabetes, cardiovascular diseases, cancer, autoimmune conditions and infectious diseases. Meta-analyses have demonstrated that serum 25(OH)D concentrations are an important predictor of survival in people with a wide variety of illnesses and have been linked to all-cause mortality in the general human population.

The role of vitamin D in non-skeletal disorders in cats and dogs is poorly understood. This is surprising since cats and dogs could act as excellent models for probing the biology of vitamin D. Vitamin D status in people is largely dependent on cutaneous production of vitamin D. This is influenced by many factors such as season, latitude and exposure to ultraviolet (UV) radiation. The interpretation of human studies investigating the effects vitamin D status on disease outcomes are therefore influenced by a number of confounding variables. Unlike humans, domesticated cats and dogs do not produce vitamin D cutaneously and obtain vitamin D only from their diet. The physiological functions and regulation of vitamin D are otherwise similar to humans. Most pets are fed commercial diets containing a relatively standard amount of vitamin D. Consequently, companion animals are attractive model systems in which to examine the relationship vitamin D status and health outcomes. Furthermore, spontaneously occurring model systems which did not require disease to be induced in healthy animals would allow the numbers of animals used in scientist research to be reduced.

This thesis aimed to define vitamin D homeostasis in companion animals in three disease settings; in cats with feline immunodeficiency virus (FIV) infection, dogs with chronic enteropathies (CE) and in hospitalised ill cats. Additional aims were to assess the prognostic significance of serum 25(OH)D concentrations in companion animals and the relationship between serum 25(OH)D concentrations and markers of inflammation. The hypothesis of this thesis was that vitamin status D would negatively correlate with presence of disease, markers of inflammation and disease outcomes. As similar findings have been demonstrated in human medicine, the hypothesis was that cats and dogs would be suitable models to investigate the role of vitamin D in human disease.

This thesis demonstrates that in dogs with a CE serum 25(OH)D concentrations are negatively correlated with inflammation and are predictive of clinical outcomes. Vitamin D status was also lower in cats with FIV and importantly vitamin D status was predictive of short term mortality in hospitalised ill cats. This research will be of interest to veterinary surgeons and opens the possibility for clinical trials which examine if low vitamin D status is causally associated with ill health and whether vitamin D supplementation results in superior treatment outcomes in companion animals. This thesis also demonstrates the potential of cats and dogs as model systems in which to examine the role of vitamin D in human health.

LAY SUMMARY

Traditionally we have understood the importance of vitamin D in maintaining bone health and preventing diseases such as rickets. However, in recent years there has been a greater understanding about the other roles played by vitamin D in maintaining good health. These include regulating immune function and inflammation, heart function, the activity of a number of hormones and maintaining normal blood pressure.

In human medicine, vitamin D deficiency is a common finding in patients with a number of different diseases and has been shown to be predictive of clinical outcomes such as treatment success and the risk of mortality. Accordingly, there is a growing interest in the role of vitamin D in the treatment and prevention of many illness. Less is known about the potential role of vitamin D in diseases of cats and dogs. This information is of great interest to veterinary surgeons and pet owners, particularly in predicting health outcomes. In addition, assessing vitamin D in people is difficult as a number of variables affect vitamin D concentrations in humans. The most important of these is exposure to sunlight and dietary intake. In contrast to people, cats and dogs do not produce vitamin D as a result of sunlight exposure and tend to consume commercial pet food supplemented with standard amounts of vitamin D. Therefore, exploring the effects of blood vitamin D concentrations on health outcomes in cats and dogs may provide a model to better understand the effect of vitamin D on human illness.

The aim of this research was to explore the association between vitamin D and infectious diseases by studying cats with feline immunodeficiency virus, to examine the relationship between vitamin D and all-cause mortality in a population of sick cats and to investigate the relationship between vitamin D status on the risk of mortality and markers of inflammation

in dogs with a chronic enteropathy. The results showed that cats infected with feline immunodeficiency virus had lower vitamin D status than healthy cats. The research also found that vitamin D status was predictive of mortality in hospitalised ill cats and of poor clinical outcome in dogs with a chronic enteropathy. Again, like in human medicine, an association between low 25(OH)D status and markers of inflammation was observed in dogs with a chronic enteropathy. These results highlight the potential for investigating the effects of vitamin on health outcomes in companion animal models of spontaneously occurring disease.

CHAPTER ONE

Thesis Overview

1.1 Introduction

Traditionally vitamin D biology has been understood in terms of its importance in maintaining skeletal health and serum ionised calcium concentrations via the actions of vitamin D receptors in the intestines, bones and kidney. In recent decades it has been appreciated that the vitamin D receptor is found in most tissues [1]. Additionally, many tissues also contain the enzyme CYP27B1[1]. This enzyme converts vitamin D to its active form calcitriol. As a result there is an increasing body of research dedicated to exploring the non-classical actions of vitamin D, including the potential associations between serum vitamin D concentrations and a number of medical conditions as well as possible clinical applications for vitamin D analogues. Low serum vitamin D concentrations, which are typically assessed by measuring the vitamin D metabolite 25-hydroxyvitamin D (25(OH)D), have been associated with a number of illnesses and health outcomes in human medicine [2]. Less research has been performed in companion animal species. Low serum 25(OH)D concentrations have been documented in dogs diagnosed with: congestive heart failure [3], spirocercosis [4], protein losing enteropathy [5, 6], renal disease [7] and neoplasia [8, 9]. Low vitamin D status has been reported in cats with inflammatory bowel disease or small cell intestinal lymphoma [10] and in feline mycobacterial infections [11]. Despite evidence that serum 25(OH)D concentrations are reduced in a number of canine and feline diseases, the clinical significance of low 25(OH)D concentrations in companion animal practice and how disease influences serum vitamin D concentrations is poorly understood. Currently knowledge regarding the significance of low serum 25(OH)D concentrations in companion animal medicine is limited to studies which have demonstrated that lower serum vitamin D concentrations are associated with poorer treatment responses in dogs treated for atopic dermatitis [12] and poor outcomes in dogs with congestive heart failure [3]. Before

considering the possible benefits of vitamin D supplementation to cats and dogs with hypovitaminosis D, there is a need for further assessment of the potential importance of vitamin D in maintaining the health of cats and dogs.

1.2 Overview of Vitamin D Biology

Vitamin D activation

The active form of vitamin D is a steroid hormone [13]. In people, the pro-hormone vitamin D3 (Cholecalciferol) is synthesised cutaneously when the cholesterol metabolite 7-dehydrocholesterol is exposed to ultraviolet (UV) light [14]. Alternatively, exogenous vitamin D3 can be obtained in the diet from sources such as oily fish and vitamin D2 can be obtained in the diet from some vegetable sources [15]. Unlike people, cats and dogs are unable to produce vitamin D cutaneously and are therefore dependent on obtaining dietary vitamin D3 from meat sources [16]. The inability of cats to synthesise vitamin D is the result of low cutaneous concentrations of the vitamin D precursor 7-dehydrocholesterol, which in turn is due to an increase in activity of the enzyme 7-dehydrocholesterol reductase [17]. Similar mechanisms might account for the lack of cutaneous vitamin D synthesis in dogs.

Both vitamin D2 and vitamin D3 are inactive pre-hormones. Activation requires further metabolism to form the biologically active form of vitamin D, calcitriol (1,25(OH)₂D) [18]. Vitamin D metabolites are transported to target tissues for further metabolism bound to vitamin D binding protein (DBP) [19, 20]. Approximately 80 to 90% of circulating 25(OH)D and calcitriol is transported bound to DBP, with 10-20% being bound to albumin. Just 0.02–0.05% of 25(OH)D circulates unbound [21]. The first step in activating vitamin D occurs in the liver, where vitamin D3 or D2 undergoes hydroxylation to form 25-

hydroxyvitamin D (25OH D) [19]. This step is largely controlled by the mitochondrial enzyme CYP27A1 [1]. The action of this enzyme is unregulated and is substrate dependent [1]. Therefore, 25(OH)D is a reliable indicator of combined dietary intake and cutaneous synthesis of vitamin D [20]. 25-hydroxyvitamin D is then transported to the kidneys where it is subsequently converted to calcitriol [22]. DBP-bound 25(OH)D, binds to megalin receptors on the proximal renal tubule cell plasma membrane and is transported into the cell for hydroxylation to occur [23]. This hydroxylation step is catalysed by the mitochondrial enzyme 25-hydroxyvitamin D-1 α -hydroxylase (CYP27B1) [19]. Although the kidney is the major site of calcitriol synthesis, CYP27B1 is found in a number of extra-renal tissues where it acts to synthesise calcitriol for local consumption as an intracrine or paracrine factor [1].

The activity and transcription of renal *CYP27B1* is stimulated by parathyroid hormone (PTH) and inhibited by fibroblast growth factor-23 (FGF-23) and calcitriol [19, 24, 25]. The renal synthesis of calcitriol can also be stimulated by calcitonin for non-calcaemic needs when normocalcaemic [26]. Control of calcitriol production in extra-renal tissues is not affected by these hormones [1]. The release of PTH is regulated by serum ionised calcium and serum phosphorus concentrations and calcitriol [27]. PTH is primarily released in response to hypocalcaemia but may also be induced by hyperphosphatemia [28]. Calcitriol acts on the intestines and kidneys to promote the uptake of dietary calcium and phosphate and reabsorption of calcium from the glomerular filtrate in the kidneys. Rising calcitriol concentrations reduce further calcitriol production by inhibiting CYP27B1 activity and activating the enzyme responsible for calcitriol catabolism; 24(OH)ase (CYP24) [19]. CYP24 can also hydroxylate 25(OH)D, reducing the pool of 25(OH)D available for activation to calcitriol [19]. CYP24 metabolises calcitriol to form inactive metabolites including 24,25-dihydroxyvitamin D (24,25(OH)₂D) and 1,24,25(OH)₃D which leads to the production of calcitroic acid [19]. CYP24 is an extremely active enzyme, however, it is

inactivated in vitamin D deficiency as transcription of the gene coding for CYP24 is activated by calcitriol [18]. This ensures that calcitriol metabolism only occurs when sufficient calcitriol is available. Calcitriol also negatively controls its own synthesis by negatively regulating PTH secretion. Calcitriol may inhibit the synthesis of PTH via conversion of 25(OH)D to calcitriol within the parathyroid gland itself and by directly activating VDR receptors on the parathyroid gland [29, 30]. Fibroblast growth factor-23, is a phosphaturic hormone which can also inhibits calcitriol synthesis. As calcitriol increases phosphate absorption, FGF-23 therefore, acts to reduce phosphate concentrations via suppression of CYP21B1 and activation of CYP24 [19].

Assessment of vitamin D status

It is possible to measure a number of vitamin D metabolites. However, vitamin D status is typically only assessed by measuring 25(OH)D concentrations [31]. Although calcitriol is the active form of vitamin D, assessing calcitriol concentrations does not provide accurate information regarding vitamin D sufficiency or deficiency. This is because, in the face of vitamin D deficiency, falling calcium concentrations will result in PTH activation. This will increase calcitriol concentrations in an attempt to maintain normocalcaemia. Therefore, even in the face of vitamin D deficiency, calcitriol concentrations in humans may be normal or elevated [31]. Furthermore, assessment of serum calcitriol levels is difficult because the half-life of calcitriol is 4-6 hours, and circulating calcitriol concentrations are a thousand fold less than 25(OH)D [31]. Unlike calcitriol production, the hepatic production of 25(OH)D is not actively regulated, meaning that serum 25(OH)D directly reflects either dietary vitamin D intake or cutaneous synthesis [31]. The half-life of 25(OH)D is life of approximately 2–3 weeks reflecting vitamin D intake and synthesis over this period of time [31].

Therefore, 25(OH)D concentrations provide the most accurate assessment of vitamin D status [31].

The vitamin D receptor

Like other steroid hormones, the action of calcitriol is mediated via nuclear hormone receptors. The vitamin D receptor (VDR) is a member of the superfamily of nuclear hormone receptors which also includes receptors for thyroid hormones and retinoic acid [1]. The VDR is found across a number of cell types, where it typically forms a heterodimer with the retinoid X-receptor in order to interact with vitamin D responsive elements (VDRE) [1, 32]. Vitamin D responsive elements are DNA sequences found in the promoter region of genes regulated by vitamin D [1]. When calcitriol binds to the VDR conformational changes occur allowing binding of VDR-complexes to VDRE resulting in expression or repression of specific genes [1]. Transcription requires other molecules to act as co-activators or co-repressors. Co-repressors block VDR mediated gene transcription in the absence of calcitriol and are displaced when calcitriol binding recruits co-activators to the VDR [1]. The VDR can also exert non-genomic actions at caveolae in cell membranes [32]. Binding of calcitriol to membrane associated VDRs activates second messenger systems, such as G protein-coupled receptors, phosphatidylinositol-3'-kinase (PI3K), phospholipase C, or protein kinase C (PKC). This can lead to a number of outcomes such as opening of voltage gated channels for electrolytes, thus allowing vitamin D to also exert rapid effects on cells [32].

Calcaemic and skeletal actions of vitamin D

Calcitriol acts alongside parathyroid hormone to maintain adequate serum ionised calcium and phosphorus concentrations for bone mineralisation. Maintaining serum ionised calcium concentrations also allows normal neuromuscular function [23]. When sufficient dietary

calcium is available vitamin D increases the uptake of dietary calcium to maintain normocalcaemia. This is achieved by the action of calcitriol on intestinal epithelial cells where it promotes the expression of calcium channels, calcium ATPase pumps and calcium-binding protein [33, 34]. The absorption of phosphate is also increased as calcitriol induces expression of the sodium-phosphate co-transporter required for intestinal phosphate absorption [35]. Calcitriol also facilitates reabsorption of calcium and phosphorus from the glomerular filtrate in the renal distal tubule [36]. When insufficient dietary calcium is available or when insufficient vitamin D is available to promote the absorption of dietary calcium, mobilization of calcium and phosphorus from bone can occur [18]. Vitamin D deficiency results in bone disorders such as rickets, osteomalacia and osteoporosis [25, 37]. Calcitriol and PTH are reported to promote the differentiation of osteoclasts to increase bone resorption [38, 39]. This is achieved by inducing osteoblasts to express receptor activator nuclear factor- κ B ligand (RANKL). Osteoclast precursors express RANK receptors which bind to RANKL on osteoblasts [23, 40]. Binding of RANK receptors to RANKL causes the differentiation and activation of osteoclasts, promoting bone resorption by releasing hydrochloric acid and collagenases thus increasing serum calcium concentrations [23, 40]. Therefore, in times of profound calcium deficiency vitamin D could act alongside PTH to promote bone resorption to maintain serum calcium concentrations. However, supra-physiological doses of calcitriol are required for osteoclast differentiation to occur and evidence from *in-vivo* experiments suggests that typically vitamin D actually inhibits bone resorption [38, 39]. *In-vivo*, bone resorption is largely regulated by PTH. In general the effects of vitamin D are to reduce bone resorption as calcitriol inhibits the release of PTH and therefore antagonises PTH-induced bone resorption [38]. Calcitriol also regulates bone development and bone health by stimulating osteoblasts to produce ALP, collagen, oestocalcin and oestopontin [41].

Non-skeletal actions of vitamin D

The VDR receptor is found on numerous cell types unrelated to calcium and phosphate homeostasis [36]. Many cells contain express CYP21B, which when activated results in local calcitriol production [1]. The local activation of vitamin D likely allows intracrine and paracrine messaging for the local regulation of cell functions and tissue growth [1].

Important actions of vitamin D beyond its effects on calcium balance includes regulation of the immune system, regulation of cell proliferation and differentiation, regulation of secretion of insulin and cardiovascular functions as discussed below.

Vitamin D and immune functions

Within the immune system vitamin D regulates cell proliferation, cellular differentiation and immunological functions to maintain immune tolerance and to promote protective immunity [42]. Within the acquired immune system, vitamin D can directly and indirectly act on lymphocytes to influence immune cells responses. Both T and B lymphocytes express VDR, and vitamin D acts to inhibit lymphocyte proliferation [43, 44]. T-helper lymphocytes (CD4⁺ T cells) modulate the function of other immune cells by releasing cytokines in response to specific antigens. The development of CD4⁺ T cells into: Th1 cells (which promote cellular immunity), Th 2 cells (which promote humoral immunity by stimulating B cells to secrete immunoglobulin), Th17 cells (which help protect against fungal pathogens and specific extracellular bacteria), or immunotolerant T-regulatory cells (which function to modulate the immune system in order maintain tolerance to harmless self-antigens and regulate the inflammatory actions of effector T-cells) determines the phenotype of an immune response. Vitamin D can alter T-helper lymphocyte phenotypes, causing a shift away from Th1 (cellular immunity) to a Th2 phenotype (humoral immunity), inhibiting

inflammatory Th17 responses and promoting the induction of T-regulatory cells [42, 45, 46]. Calcitriol also down regulates harmful, pro-inflammatory immune responses by auto-reactive Th-17 lymphocytes [47]. Cytotoxic T cells, which are directly involved in cell mediated immunity, also express the VDR, but calcitriol has less of an anti-proliferative effect on CD8+ cells [48]. However, calcitriol activity regulates cytotoxic T-cell cytokine production [49] and regulates the proliferation of cytotoxic T-cells in response to certain stimuli [50]. Vitamin D also regulates antibody producing B-cells by limiting on going proliferation, differentiation and secretion of immunoglobulin by activated B-cells and inducing B-cell apoptosis [44, 51].

Dendritic cells are antigen-presenting cells. The main function of dendritic cells is to process antigenic material and present it to T-lymphocytes. Cytokines produced by antigen presenting cells directs the phenotype of T-cells responses. Antigen presenting cells are key targets for the immunomodulatory effects of calcitriol [52]. Studies have consistently demonstrated that vitamin D inhibits the maturation of dendritic cells [45, 53, 54]. Vitamin D promotes the induction of tolerogenic dendritic cells which promote regulatory T-cell responses. This is achieved by down-regulating the expression of the co-stimulatory molecules CD40, CD80 and CD86, which are required for T cell activation, by reducing the expression of inflammatory cytokines such as interleukin-12 (IL-12) and by increasing production of the anti-inflammatory cytokine IL-10 [52]. Vitamin D-treated dendritic cells can also induce auto-reactive T-cell apoptosis [55]. Vitamin D has additionally been shown to increase the expression of immunoglobulin-like transcript 3 (ILT3) [52]. This protein belongs to a family of inhibitory receptors expressed by human monocytes and dendritic cells which have been shown to negatively regulate the activity of antigen presenting cells [56]. As calcitriol promotes up-regulation of inhibitor ILTs this may be an additional

mechanism by which vitamin D can promote immune tolerance [52, 56]. Therefore, the combined actions of vitamin D on the acquired immune system are to promote generation of tolerogenic rather than immunogenic dendritic cells, to promote a regulatory T cell phenotype and to suppress the inflammatory effects of effector T cell functions.

Vitamin D also exerts effects on the innate immune system. Vitamin D is known to enhance the innate immune response to bacteria. Antigen binding to toll-like receptors on innate immune cells increases expression of the VDR and CYP27B1 [57]. The local availability of activated vitamin D-VDR complexes is therefore increased during innate immune responses. This allows vitamin D-VDR complexes to bind to VDRE in target genes within these cells [42]. Genes regulated by VDRE include those associated with the production of anti-microbial peptides such as cathelicidin [57-59]. Vitamin D can therefore enhance the anti-bacterial functions of innate immune cells in response to antigenic stimulation. Vitamin D also has a negative feedback role on innate immune cells activity. For example, in macrophages the local production of calcitriol leads to down-regulation of toll-like receptor signalling, preventing excessive immune responses [60]. Local vitamin D production has also been demonstrated to reduce pro-inflammatory cytokine production in monocytes and macrophages [61]. In addition to its functions on leukocytes, vitamin D may also modulate innate immunity by its effects on barrier function at mucosal and epithelial surfaces. Epithelial cells also contain CYP27B1 [62, 63]. Vitamin D receptors may therefore function to maintain protective barrier functions at these sites. For example, in the gastrointestinal tract calcitriol can alter the expression of tight junction proteins [64]. In the skin, vitamin D is also important in the production of lipids that play a role in barrier function [62].

Given the known functions of vitamin D in the immune system, numerous studies have investigated the association between vitamin D status and both autoimmune diseases and infectious diseases. Observational studies have demonstrated that patients with vitamin D deficiency could be at greater risk for developing immune-mediated diseases [65].

Associations between low vitamin D status and autoimmune diseases are reported for condition such as systemic lupus erythematosus and multiple sclerosis [66, 67], rheumatoid arthritis and psoriasis [68]. Vitamin D deficiency is also associated with increased autoimmune responses in healthy people [69]. However, despite these associations the design of these studies cannot conclude that the association between vitamin D status and risk of immune mediated disease is definitely a causal relationship.

The best studied link between bacterial infections and low vitamin D status is in tuberculosis (TB) infections in people [70, 71]. Not only are serum 25(OH)D concentrations low in people with TB, but supplementation with vitamin D appears to be beneficial in treating pulmonary TB infections [71, 72]. Low serum 25(OH)D concentrations are also a risk factor for infections such as pneumonia and acute lower respiratory infections [73, 74], sepsis [74] and *Clostridium difficile* infections [75]. In addition low 25(OH)D status has been associated with viral infections such as influenza [76] and Human Immunodeficiency Virus (HIV) [77, 78]. Vitamin D deficiency is associated with poor disease outcomes in viral infections such as chronic hepatitis B [79] and hepatitis C infections [80]. Additionally, sub-optimal vitamin D status and subnormal calcitriol concentrations have been associated with lower CD4 + lymphocyte counts in HIV infected people and HIV progression and acquired immune deficiency syndrome (AIDs) related morbidity and survival [81-84]. Therefore, a predisposition to infectious diseases or poorer immune responses to infections may occur in individuals with a low vitamin D status, although this has not been definitely demonstrated.

Vitamin D and endocrine functions

Vitamin D has been shown to be important in the regulation of hormone secretion and endocrine functions. The regulation of PTH secretion is important for both bone and calcium homeostasis. However, vitamin D also influences the secretion of other hormones. It has been demonstrated that vitamin D can stimulate beta pancreatic cells to secrete insulin [85-87] and vitamin D receptor knock-out mice have reduced insulin secretory capacity [88]. Vitamin D deficiency has also been related to impaired insulin action in type 2 diabetes mellitus, metabolic syndrome and obesity [89-91]. Furthermore poor vitamin D status is epidemiologically associated with diabetes mellitus [92, 93]. The renin-angiotensin system (RAAS) is also regulated by vitamin D. This has been demonstrated in VDR knock out mice, where excessive renin production is associated with hypertension and cardiac hypertrophy [94-96]. Inappropriate activation of the RAAS can result in hypertension. Observational studies have reported an association between vitamin D-deficiency and high blood pressure [97, 98]. The effects of vitamin D on regulation of the renin-angiotensin system may explain the epidemiological association between subnormal vitamin D concentrations and hypertension in people. This hypothesis is supported by evidence of higher circulating angiotensin concentrations in vitamin D insufficient people [99]. There appears to be a small benefit in supplementing hypertensive patients with vitamin D [100].

Vitamin D and cardiovascular disease

In addition to regulating the renin-angiotensin system, which is important for cardiac health, there is also evidence that vitamin D has physiological effects on cardiomyocytes, vascular smooth muscle and the vascular endothelium [101]. Murine experiments have demonstrated that vitamin D has important effects in modulating and maintaining cardiac cell structure and

function [102]. Secondary hyperparathyroidism is associated with vitamin D deficiency. Excessive PTH concentrations are related to increased blood pressure and increased cardiac contractility, leading to cardiomyocyte hypertrophy and fibrosis [103]. Meta-analyses of several observational studies suggests that there is an inverse association between serum 25(OH)D concentrations and the risk of cardiovascular disease and death due to cardiovascular disease [104, 105]. Vitamin D concentrations are also predictive for the incidence of cardiovascular disease and death over a 13 year period [106]. Poor 25(OH)D status is also commonly seen in patients with congestive heart failure [107]. In veterinary medicine, an association between congestive heart failure and low serum 25(OH)D concentrations has been reported in dogs [3]. This study also demonstrated that like in human medicine, patients with low serum 25(OH)D concentrations had poorer outcomes. In addition to its reported effects on cardiac function and the RAAS, vitamin D may also be protective in cardiovascular disease due to its previously discussed effects in modulating inflammation and the immune system [62].

Vitamin D and cancer

In vitro and *in vivo* studies indicate that calcitriol promotes cell differentiation, regulates cell proliferation, promotes cellular apoptosis and has antiangiogenic properties [33]. These functions therefore provide a mechanistic explanation as to how vitamin D could prevent the development of cancer. Vitamin D can block a number of pathways associated with cellular proliferation such as the β -catenin, MAP kinase 5 (MAPK5), nuclear factor κ B (NF- κ B) and transforming growth factor β (TGF β) [33, 108]. Calcitriol can also activate cyclin-dependent kinase inhibitors such as p21 and p27; inhibit growth factors including insulin like growth factor 1 (IGF-I) and epidermal growth factor receptor [108, 109] and can promote expression of growth regulatory genes such as TGF- β [110]. Furthermore calcitriol induces

apoptosis by down-regulating anti-apoptotic genes and up-regulating pro-apoptotic genes [111, 112]. The anti-inflammatory effects of calcitriol decrease prostaglandin synthesis which is required for tumour angiogenesis and metastasis implicating sub-normal vitamin D concentrations in the development of neoplasia [113, 114]. Due to its effects on apoptosis and cellular proliferation, the potential for vitamin D to influence the development, outcome and treatment of neoplastic diseases has received a great deal of attention. Numerous studies have documented an association between low vitamin D status and many types of cancer [115-118]. Vitamin D supplementation may have some benefits in cancer prevention [119], although this has not been universally reported from clinical trials [120, 121]. Low serum 25(OH)D concentrations have also been reported in cats with intestinal small cell lymphoma [10], dogs with mast cell tumours [9] and dogs with splenic neoplasms [8].

Vitamin D and muscle function

Vitamin D may be important for muscle function. It has been suggested that binding of calcitriol to the nuclear VDR in skeletal muscle results in the synthesis of muscle proteins [122]. This hypothesis is supported by studies which show an increase in type II muscle fibres after treatment with vitamin D [123]. Vitamin D may also have non-genomic effects on muscle tissues via effects on calcium signalling and the sarcoplasmic reticulum [124]. Observational investigations have demonstrated a positive association between higher 25(OH)D status and muscle strength [125]. A meta-analysis of 8 randomized interventional studies shows that vitamin D supplementation lowered the incidence of falls in older people by 72% compared to those receiving a placebo [126], suggesting vitamin D can increase muscle strength in this population.

Vitamin D and renal disease

Low 25(OH)D concentrations are common in patients with chronic kidney disease and plasma 25(OH)D concentrations are an independent predictor of disease progression and death in patients with chronic kidney disease [127, 128]. Supplementation with exogenous vitamin D appears to offer survival benefits [129, 130]. The relationship between calcitriol and the renin-angiotensin system may help explain the association between low serum 25(OH)D concentrations and poor outcomes in renal disease. Causes of low serum 25(OH)D concentrations in patients with chronic kidney disease may include decreases in megalin, which is the binding protein for vitamin D found in the proximal tubule of the kidney. A decrease in megalin associated with reduced renal mass may reduce renal reabsorption of vitamin D [131]. In addition, vitamin D and vitamin D binding protein are lost in protein losing nephropathies [132]. Furthermore, impaired renal function and reduced renal mass limit the amount CYP2B1 available for production of the active vitamin D metabolites [133]. Phosphate retention associated with declining renal function will also increase FGF-23 concentrations and down regulate the conversion of 25(OH)D to calcitriol [133, 134]. Like in people, serum 25(OH)D concentrations are also lower in dogs with chronic kidney disease [7].

Low Serum Vitamin D concentrations: cause or consequence of disease?

It is clear that vitamin D exerts pleiotropic effects on a number of cells types and therefore, low vitamin D concentrations could conceivably contribute to ill health in a number of diseases. Not only have low serum 25(OH)D concentrations been associated with a number of diseases but poor vitamin D status has also been linked to all-cause mortality in the general human population [44] and in a population of sick foals [135]. Meta-analyses have demonstrated that serum 25(OH)D concentrations are an important predictor of survival in

people with a wide variety of illnesses [45-48]. However, it is unclear if low serum vitamin D concentrations are cause or consequence of ill health. Despite the association between low vitamin D status and numerous non-skeletal diseases, meta-analyses of randomised controlled trials investigating the effects of vitamin D supplementation often do not support the hypothesis that vitamin D concentrations are causally associated with disease risk or health outcomes [105, 136]. Factors associated with poor health such as poor diets and a lack of dietary vitamin D [137-139], lack of exposure to sunlight [140-142] and obesity [143, 144] are all associated with poor vitamin D status. Therefore, low vitamin D status may be a marker for other causes of ill health. Furthermore, lower vitamin D concentrations are common with increasing age [145, 146] and the prevalence of many diseases would be expected to be higher in elderly individuals.

Recent research suggests that sunlight exposure may have other health benefits beyond stimulating the production of 25(OH)D in human skin. Lui et al have demonstrated that exposure to UV light reduces blood pressure and the authors propose that this is linked to the cutaneous production of nitric oxide [147]. Therefore, serum 25(OH)D concentrations may be a biomarker for other benefits of sunlight exposure and the apparent associations between serum 25(OH)D concentrations and a number of diseases may not be directly related to vitamin D status. Clearly, as cats and dogs do not cutaneously produce vitamin D, the association between serum 25(OH)D concentrations and diseases in companion animals is unlikely to be related to sunlight exposure.

As low vitamin D status is commonly reported in a number of inflammatory diseases, it is hypothesised that low serum vitamin D concentrations are a consequence of inflammation rather than a cause of inflammatory illnesses. Serum 25(OH)D concentrations have been shown to decrease following elective surgical procedures, coupled with increases in inflammatory markers such as C-reactive protein (CRP), suggesting that vitamin D is a

negative acute phase reactant [148]. Increases in pro-inflammatory cytokines have been proposed as the cause of decreases in serum 25(OH)D seen in acute inflammation. For example, declining serum 25(OH)D concentrations over several weeks following elective knee arthroplasty corresponded with increases in serum pro-inflammatory cytokines [149].

Assessing the role of vitamin D on human health outcomes is hampered by a large number of environmental variables which influence serum vitamin 25(OH)D concentrations. These include factors such as ethnicity [139, 150, 151], dietary intake of vitamin D [137-139], seasonality [152], latitude and exposure to sunlight [140-142], obesity [143, 144], age [145] and gender [153]. Exploring the relationship between serum 25(OH)D concentrations and health outcomes in a model system in which many of these confounding factors are avoided would be of significant interest. Furthermore, a model system which did not require disease to be induced in otherwise healthy animals would allow the number of animals used in scientific research to be reduced. Most cats and dogs eat a commercial diet which is supplemented with a similar amount of vitamin D [154]. In addition, cats and dogs do not synthesize vitamin D cutaneously meaning that serum 25(OH)D concentrations are not influenced by exposure to UV radiation and that vitamin D is not biomarker for other beneficial effects of sunlight exposure [16, 17]. Therefore, as fewer confounding variables affect vitamin D assessment in cats and dogs, companion animal could provide a novel model system in which to investigate the health benefits of vitamin D.

1.3 Thesis Objectives

The importance of vitamin D in maintaining human health is unclear. Even less is known about the clinical importance of low serum 25(OH)D concentrations in ill cats and dogs, despite low 25(OH)D having been reported in a number of companion animals diseases. The general objective of this thesis was to define vitamin D homeostasis in companion animals by investigating serum 25(OH)D concentrations in hospitalised ill cats, dogs with chronic enteropathies (CE) and cats with feline immunodeficiency virus (FIV) infection. The aim was to assess the prognostic significance of serum 25(OH)D concentrations in companion animals and to assess the possible relationship between serum vitamin D concentrations and inflammation. Furthermore, if the findings of this thesis mirrored those seen in human practice, then this thesis aimed to assess if cats and dogs could provide a model system in which to investigate the effects of 25(OH)D concentrations on human health.

CHAPTER 2

Investigation of vitamin D status as a predictor of 30 day mortality in hospitalised cats

2.1 Introduction

Vitamin D insufficiency has been associated with a number of disorders in people including hypertension [155], diabetes mellitus [156], cardiovascular diseases [104], cancer [116], autoimmune conditions [157] and infectious diseases [83, 158, 159]. Furthermore, low serum 25(OH)D concentrations have also been linked to all-cause mortality in the general human population [160]. Meta-analyses have demonstrated that serum 25(OH)D concentrations are an important predictor of survival in people with a wide variety of illnesses [105, 161-163]. One of the most striking findings is the negative association between serum 25(OH)D concentrations and all-cause mortality. This finding was consistent across study populations, sexes, age groups and seasons [161, 162].

Despite the associations between mortality and lower serum 25(OH)D concentrations, many randomised intervention trials with vitamin D supplements have found little effect on mortality, suggesting that low serum vitamin D concentrations may be a consequence of serious illness rather than being causally associated with mortality [164, 165]. However, one meta-analysis has demonstrated that intakes of normal amounts of vitamin D are associated with reduced mortality [166] and another recent meta-analysis also showed that vitamin D supplementation reduced mortality in elderly people [167]. Higher 25(OH)D concentrations may also be associated with an increased risk of mortality. In a population of patients hospitalised in intensive care, supra-physiological serum concentrations of 25(OH)D were associated with an increased risk of death [168]. Other studies have demonstrated similar findings. For example, in an observational study investigating the effect of pre-

hospitalisation serum 25(OH)D concentrations in patients admitted to two American hospitals, the risk of all-cause mortality 90 days after admission to hospital was greater for patients with serum 25(OH)D concentration less than 20 ng/mL and greater than 60ng/mL [169]. Similar findings have been reported in the general population in the United States [170], in primary care patients in Copenhagen [171], in elderly women [172] and in elderly men [173]. The association between all-cause mortality and elevated vitamin D concentrations has not been consistently reported. This may be due differences in study design between epidemiological studies investigating the prognostic significance of serum 25(OH)D concentrations. Many of the studies examined the effects of vitamin D as a non-continuous variable based on quintiles, quartiles or tertiles [105, 161, 174, 175]. By grouping very high serum 25(OH)D concentrations with more moderate concentrations the association with higher vitamin D status may have been lost in these studies. Furthermore, in many studies, relatively few patients had very high serum vitamin D concentrations, meaning many studies may have been underpowered to examine the effect of higher serum vitamin D concentrations.

A large number of factors are known to influence serum 25(OH)D concentrations in people. These factors include: ethnicity [139, 150, 151], diet [137-139], seasonality [152], latitude and exposure to sunlight [140-142], obesity [143, 144], age [145] and gender [153]. This makes interpreting the results of observational studies documenting an association between serum 25(OH)D and mortality in humans particularly challenging. Investigating the relationship between serum 25(OH)D concentrations and all-cause mortality in a model mammalian system in which many of these confounding factors are avoided would be of significant interest. Advantages of investigating the role of 25(OH)D on health outcomes in cats include a more standard dietary intake of vitamin D since almost all cats which attended the referral veterinary hospital in this study eat a commercial diet which is supplemented with a similar amount of vitamin D [154]. In addition, cats do not synthesise vitamin D

cutaneously meaning that serum 25(OH)D concentrations are not influenced by exposure to UV radiation [16, 17].

Investigating the role of serum 25(OH)D concentrations and all-cause mortality in cats would be of interest to veterinary surgeons since it is presently difficult to accurately predict mortality in hospitalised ill cats. The identification of clinical measures which were predictive of mortality would be extremely helpful in providing much needed prognostic information to owners of ill cats.

Hypothesis

The hypothesis of this study was that cats with lower serum 25(OH)D concentrations would have higher mortality at 30 days post admission to hospital than cats which were vitamin D replete. This would make client owned, domesticated cats which acquired spontaneous diseases a suitable model in which to study the relationship between serum 25(OH)D concentrations and all-cause mortality in people.

Objectives

The objectives of this study were to;

- Measure serum 25(OH)D concentrations in a general hospital population of ill cats and to assess if serum 25(OH)D concentrations were a predictor of short term (30 day) all-cause mortality.
- Measure other clinical variables including; age, sex, breed, appetite scores, serum albumin, creatinine, potassium, sodium, total calcium concentrations, total packed cell volume and total white cell counts to investigate their ability to predict short

term outcomes in ill cats. The potential of these factors to confound any relationship between mortality and serum 25(OH)D concentrations in cats was also investigated.

2.2 Material and Methods

Study Population

Consecutive cats examined at the Royal (Dick) School of Veterinary Studies, Hospital for Small Animals were considered eligible for inclusion in the study. Informed consent for the use of residual clinical blood samples for research purposes was obtained at admission for each cat enrolled. Ethical approval for the study was obtained from the University of Edinburgh's Veterinary Ethical Review Committee.

Clinical records were reviewed for each cat enrolled. The age, sex and breed were recorded for each cat. Survival data was obtained from clinical records or follow up telephone calls to clients and referring veterinary surgeons for each cat at day 30 after first presentation to the Hospital for Small Animals. The appetite of the cats was graded as normal or reduced. The following clinical information was extracted from the clinical records of each cat enrolled: white blood cell count, packed cell volume (PCV), serum albumin concentrations, serum creatinine concentration, serum sodium concentrations, serum potassium concentrations and total serum calcium concentration. Haematology variables were measured on an ADVIA(r) 2120i System with Autoslide (Siemens Medical Solutions Diagnostics Ltd California, USA). Biochemistry parameters (serum sodium, potassium, creatinine, albumin and total calcium) concentrations were measured on an ILab650 biochemistry analyser, (Diamond Diagnostics, USA).

Following handling of blood samples for routine diagnostic procedures, serum samples were stored initially at -20°C and later moved to -70°C for longer term storage until 25(OH)D concentrations were measured as a batch. Previous studies have indicated that 25(OH)D is stable when stored at -20°C [176]. Serum concentrations of 25(OH)D₂ and 25(OH)D₃ were determined by liquid chromatography tandem mass spectrophotometry (LC-MS/MS) using an ABSciex 5500 tandem mass spectrophotometer (Warrington, UK) and the Chromsystems (Munich, Germany) 25OHD kit for LC-MS/MS following the manufacturers' instructions (intra- and inter-assay CV 3.7% and 4.8% respectively). This Supraregional Assay Service laboratory is accredited by CPA UK (CPA number 0865) and has been certified as proficient by the international vitamin D Quality Assurance Scheme (DEQAS). Total 25(OH)D is defined as the sum of 25(OH)D₂ and 25(OH)D₃. The laboratory measuring the vitamin D metabolites was blinded to the clinical data from the enrolled cats. In addition, veterinary surgeons caring for the enrolled cats were not aware of 25(OH)D results during the clinical management of the cats.

Statistical Analysis

Initially, 25(OH)D concentrations were compared between cats which died within 30 days of sampling to cats which survived 30 days by a Mann-Whitney U test. In order to investigate for the presence of confounding variables a standard binary logistic regression model of death by 30 days was constructed. A range of clinical and biochemical data including sex, age, breed, total white blood cells, packed cell volume and serum concentrations of albumin, total calcium, creatinine, sodium and potassium were included, as was an assessment of appetite as a binary variable (normal or reduced). Initially 25(OH)D concentrations were included as a linear predictor within the logistical regression model. Serum 25(OH)D concentrations were also divided into 3 categories based on 33% and 66% tertiles treating the variable as a three level factor and also as low versus middle and high categories

combined. Akaike's information criteria (AIC – a parameter penalised measure of model fit) was used to stepwise select variables which were to be retained to identify a final model with minimum AIC (i.e. best parameter penalised fit). P values for individual variables were calculated using Wald's test. A p-value of < 0.05 was considered to be evidence of statistical significance.

2.3 Results

A total of 99 cats were recruited to the study. The median age of the cats was 96 months. There were 3 intact males, 56 neutered males, 1 intact female and 39 neutered female cats. Breeds enrolled in the study included; 62 Domestic Short Hairs, 8 Domestic Long Hairs, 6 Maine Coons, 5 Burmese, 3 Bengals, 2 Tonkinese, 2 Siamese, 2 Ragdolls, 2 Burmese crosses, 2 Oriental Short Hairs, 1 Manx Cat, 1 British Short Hair, 1 Chinchilla, 1 Burmilla and 1 Abyssinian.

There was a significant difference between the 25(OH) D concentrations of cats which were alive (n=80) compared to cats which had died at 30 days (n=19) ($p=0.0022$, fig.1).

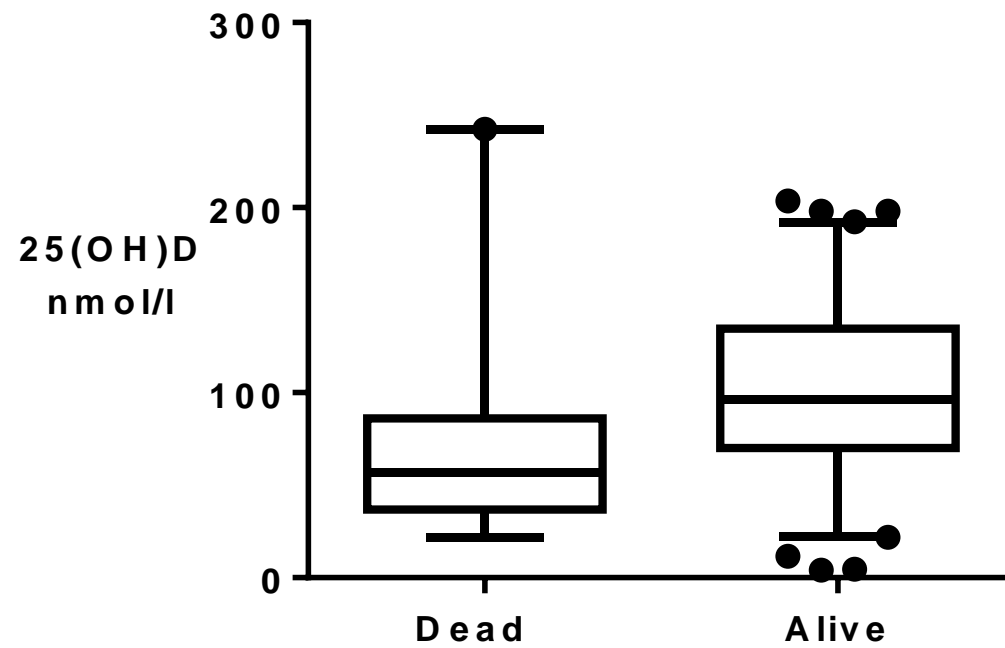


Figure 1: Box and whiskers plot of serum concentrations of 25(OH)D in cats which died or were alive at 30 days post admission. Box extends from 25th-75th percentiles with solid line representing the median value. Whiskers extend to 5th-95th percentiles

Using serum 25(OH) D concentrations as a linear predictor of survival within the logistic regression model, none of the variables, including 25(OH)D concentration, were significant predictors of mortality. Since several studies in human medicine have also shown a non-linear relationship between vitamin D status and mortality [160, 171, 177], serum 25(OH)D concentrations were also represented as a categorical variable. When serum 25(OH)D concentrations were considered as a categorical variable, cats with a 25(OH)D concentration in the lower tertile had an increased risk of mortality compared to cats in the middle tertile reference category (Table 1). There was no significant difference in survival between cats in the upper and middle tertile (Table 1). The only other parameters which were associated with an increased risk of mortality at 30 days post hospital admission were serum potassium concentrations and a reduced appetite (Table 1).

Variable	Odds ratio (95% CI)	P value
Potassium	4.23 (1.36-14.59)	0.0153
Reduced appetite	4.05 (1.17-17.04)	0.0370
25(OH)D category low (<73.6nmol/l)	9.51 (2.25-57.07)	0.0051
25(OH)D category middle (73.6-110.05nmol/l)	Reference category	Reference category
25(OH)D category high (>110.05nmol/l)	1.31 (0.21-8.66)	0.7681

Table 1: Results of logistic regression model including vitamin D as three tertile categorical variable. Table only shows significant variables. (AIC = 85.50)

Based on the results of the three tertile model and the epidemiological data which links low vitamin D status to poor health outcomes [161], the middle and upper tertiles were combined into a single binary predictor in a third model. A serum 25(OH)D concentration in the lower

tertile remained predictive of 30 day mortality (Table 2). Again, serum potassium concentrations and a reduced appetite were the only other parameters included in the third model which were predictive of survival (Table 2).

Variable	Odds ratio (95% CI)	P value
Potassium	4.14 (1.34-14.21)	0.0161
Reduced appetite	4.02 (1.16-16.83)	0.0379
25(OH)D category low (<73.6nmol/l)	8.27 (2.54-31.52)	0.0008

Table 2: Results of logistic regression model combining vitamin D as a categorical variable using vitamin D as a binary predictor of lower tertile versus middle and upper tertiles combined. Table only shows significant predictors. (AIC = 83.58)

2.4 Discussion

The central finding of this study demonstrates that hospitalised ill cats with low serum 25(OH)D concentrations were less likely to survive 30 days post hospitalisation. Using a regression model which included serum 25(OH)D concentrations as a linear variable, none of the 12 clinical, biochemical and haematological parameters, including 25(OH)D concentrations, were predictive of mortality. However, when a second analysis in which serum 25(OH)D concentrations were included as a categorical variable was performed, results demonstrated that low vitamin D status was an independent predictor of short term mortality.

The finding that there was a relationship between low serum 25(OH)D concentrations and mortality is consistent with numerous human studies [161, 163, 178]. In addition, the observation that there was not a linear relationship between vitamin D status and survival is also consistent with studies in human patients [160, 171]. Several human studies have reported that there is minimal benefit of having high serum 25(OH)D concentrations and a number have linked high vitamin D status to negative health outcomes [169, 171]. There wasn't a significantly increased risk of death for cats with higher vitamin D concentrations in this study. However, the possibility that higher serum 25(OH)D concentrations could be associated with poor health outcomes, should be borne in mind when setting hypotheses in any future studies investigating the association of vitamin D and health outcomes in cats and in any prospective clinical trials employing vitamin D supplementation.

It is unclear if serum 25(OH)D concentrations are directly associated with health outcomes or are just a reflection of disease severity. Alternatively interventions and treatments commonly given to ill patients may also affect serum 25(OH)D concentrations. One study suggests reduced vitamin D concentrations, particularly in the critically ill where patients are commonly given large volumes of intravenous fluids, may be a consequence of haemodilution [179]. Many of the cats enrolled in this study may have received fluid therapy, which could have affected the results of this study. However, blood samples from the cats enrolled in this study were collected at the time of admission to the referral hospital, prior to the administration of any medical interventions. Therefore, unless the cats had been given large volumes of fluids by the referring veterinary surgeons prior to referral, haemodilution is unlikely to be a significant contributor to low serum 25(OH)D concentrations in this study. Some medications including glucocorticoids [180] and anti-epileptic drugs [181] can influence vitamin D metabolism in humans. However, it is unclear whether many commonly used drugs can influence vitamin D metabolism in companion

animals. However, it has been previously shown that glucocorticoids do not alter vitamin D metabolism in dogs [182]. As many cats would have received some form of medical treatment prior to referral, the inability to assess the impact of certain medications on vitamin D homeostasis is a limitation of this study.

Reverse causation or confounding variables could account for the association between low 25(OH)D concentrations and 30 day mortality. For example, vitamin D status could be a marker for other variables which cause poor health and increase the risk of mortality. For example, in people a link between obesity and low 25(OH)D has been reported [183, 184] and obesity is associated with increased all-cause mortality [185]. Further work is required to see if environmental or lifestyle factors could account for lower serum 25(OH)D concentrations in cats. Another potential explanation for the association between poor outcomes and vitamin D status is the hypothesis that vitamin D is a negative acute phase reactant. A negative acute phase reactant is a substance whose plasma concentrations decrease in response to inflammation. It has been shown that serum vitamin D binding protein concentrations and 25(OH)D concentrations are likely to decrease as part of the systemic inflammatory response [186]. This may explain why serum 25(OH)D concentrations tended to be lower in the cats which died as inflammatory processes may have been more marked in these cats compared to inflammatory processes in surviving cats.

Observational studies are unable to determine if low serum 25(OH)D are a cause of consequence of illness and are inherently affected by confounding variables and the possibility of reverse causality. To assess the importance of serum 25(OH)D concentrations as a predictor of mortality in human medicine, investigators have studied the effect of genetic mutations in the enzymes 7-dehydrocholesterol reductase (DHCR7) and vitamin D

25-hydroxylase (CYP2R1) on the risk of mortality [187]. Mutations in genes coding for these enzymes lead to low serum 25(OH)D concentrations [187]. Genetic variants that mirror the biological effects of a modifiable environmental exposure (such as low vitamin D status), can investigate the risk of variables on disease outcomes without being confounded by a range of other environmental, social or behavioural factors [188]. In addition, reverse causation is also avoided in these studies [188]. Research conducted by Afzal et al suggests that lower serum 25(OH)D concentrations due to genetic mutations were associated with both all-cause mortality and cancer mortality [187]. There was no association between these genetic mutations and other risk factors for mortality [187]. The authors of this study therefore concluded that these results suggest that low 25(OH)D may genuinely be causally associated with poor health outcomes.

There are many potential explanations for why low vitamin D status could causally be associated with increased all-cause mortality. One possible important factor is the role of vitamin D metabolites on immune function and inflammation. The vitamin D receptor is expressed on many immune cell types and it is clear that vitamin D can modulate both the innate and acquired immune responses via effects on monocytes, macrophages, dendritic cells and lymphocytes [189, 190]. One of the best studied links between vitamin D metabolites and immune function in critically ill patients is the association between vitamin D status and anti-bacterial immune responses. This includes the effects of calcitriol on stimulating the production of the anti-bacterial peptide cathelicidin in neutrophils, macrophages, monocytes and epidermal cells [59]. One prospective cohort study of critically ill patients found that lower total 25(OH)D levels were associated with lower human cathelicidin (hCAP18) concentrations and that lower hCAP18 levels were associated with a greater risk of both sepsis and 90-day mortality [191]. Similar changes may also occur in cats and could partially account for the association between low 25(OH)D and mortality in

the cats in this study population, particularly as some of these cats presented with serious infections such as a pyothorax.

The renin-angiotensin system is negatively regulated by vitamin D [96, 99]. Up-regulated renin-angiotensin activity is associated with systemic hypertension, renal dysfunction, vascular damage [192] and cardiac hypertrophy [193]. Feeding transgenic rats which overproduce angiotensin and renin a diet deficient in vitamin D aggravates hypertension and end target organ damage [194]. Furthermore, supplementing vitamin D deficient patients with hypertension blunts systemic renin-angiotensin activity [195]. The vitamin D receptor has also been shown to block the renin-angiotensin system in a rodent model of acute lung injury [196]. Increased renin has been associated with microvascular dysfunction and organ failure in patients with sepsis [197]. Therefore, interactions between calcitriol and the renin-angiotensin system may provide another mechanistic link to explain the association between poor vitamin D status and an increased risk of mortality.

The pleiotropic extra-skeletal effects of vitamin D extend to vascular function [198] and the response of vascular endothelium to injury [199]. Stress to the endothelium causes proliferation of vascular smooth muscle, which is inhibited by vitamin D metabolites [200]. Vascular dysfunction is also associated with an increase in thrombotic events. Vitamin D analogues have been shown *in-vitro* to down regulate the expression of pro-thrombotic proteins by human aortic smooth muscle cells [201]. As thrombosis is an important cause of mortality and morbidity in veterinary patients [202], this may also account for potential associations between serum 25(OH)D concentrations and mortality in this population of cats. Similarly cardiopulmonary dysfunction is commonly recognised as a cause of mortality. Serum 25(OH)D concentrations have been associated with lung function [203] and vitamin D is also important for cardiac function and health [204].

Hypocalcaemia has also been reported as a predictor of poor outcome in critically ill human patients [205, 206]. In feline practice, hypocalcaemia has been identified as a negative prognostic indicator in cats with pancreatitis [207]. Hypovitaminosis D may contribute to the hypocalcaemia seen in critically ill humans, although this is only typically seen with profound hypovitaminosis D [208]. However, it is possible that moderate hypovitaminosis D could contribute to hypocalcaemia alongside other risk factors associated with critical illness [209]. In this population of cats, total calcium concentrations were not associated with survival and therefore alterations in calcium status secondary to changes in serum 25(OH)D concentrations are unlikely to account for the association between lower 25(OH)D and mortality in this study. However, measurement of ionised calcium may have provided a more accurate assessment of calcium status.

This study also demonstrated that reduced appetite was an independent predictor of short term mortality in cats. This finding is similar to human studies where reduced appetite has been linked to poor health outcomes in elderly patients [210]. However, serum 25(OH)D concentrations remained a significant predictor of mortality when the results were corrected for reduced appetite. This suggests that the association between low serum vitamin D concentrations and mortality is not simply due to reduced dietary intake of vitamin D in hospitalised cats.

The study also demonstrated that potassium concentrations were linked to mortality with increasing potassium concentrations associated with poor survival outcomes. High serum potassium concentrations have been associated with mortality in critical care patients, even when increases in potassium are modest [211] and in patients with cardiac and renal disease

[212]. The mechanism(s) by which hyperkalaemia influences mortality are unclear. However raised potassium concentrations can result in altered neurological, cardiac and muscular functions [211]. Furthermore, declining renal function is also associated with hyperkalaemia [213] and hyperkalaemia has been shown to be associated with serious infections and haemorrhage [211] in people, which may in part explain its association with mortality.

In contrast to human medicine, little is known about the factors which are involved in all-cause mortality in cats. Previous studies have focused on cats admitted to an intensive care unit (ICU), rather than across a wider hospital population [214, 215]. Therefore, the use of serum 25(OH)D concentrations to predict survival in a general hospital population is an important feature of this study. A previously reported predictor of feline survival is the Feline Acute Patient Physiologic and Laboratory Evaluation (Feline APPLE) Score [215]. This scoring system has been validated only for feline ICU patients and requires several clinical and diagnostic parameters to be assessed. A univariable measure such as serum 25(OH)D concentration may provide a simpler and more readily usable predictor of mortality. This study therefore provides valuable information about a possible prognostic marker for use in feline practice.

One significant limitation of this study in assessing mortality is that ill cats may be euthanized by their owner for a number of reasons, including financial limitations. However, the cats which were euthanized in this study were generally seriously unwell, requiring tertiary referral care. Euthanasia was generally performed only when clinicians advised owners that the prognosis was very poor. Clinicians were blinded to vitamin D status at the time of hospitalisation; recommendations for euthanasia were therefore not based on serum 25(OH)D concentrations. Euthanasia is often the predominant cause of death in

veterinary patients for ethical reasons. Therefore, when assessing a potential biomarker for mortality it would be unreasonable to exclude cases which died as a result of euthanasia.

It cannot be concluded that serum 25(OH)D is causally linked to mortality from the finding that low vitamin D status is an independent risk factor for 30 day mortality in hospitalised, ill cats. This would require further prospective studies, including randomised, placebo controlled supplementation studies of cats with low vitamin D status. In light of the finding that cats with 25(OH)D concentrations in the upper tertile had a similar incidence of mortality as cats in the middle tertile, future studies could focus on assessing whether correction of hypovitaminosis D improves health outcomes. This approach is supported by observations from human trials in critically ill patients [216]. Similarly, a study investigating the effects of vitamin D on cardiovascular morbidity and mortality, revealed that although supplementation improved overall survival, the effects were only significant in vitamin D deficient patients [217].

2.4 Conclusion

In conclusion, this study supports the hypothesis that low serum vitamin D status is predictive of 30 day mortality in hospitalised cats. The finding that low serum 25(OH)D concentrations are negatively correlated with survival supports the initiation of follow up clinical trials to examine the influence of vitamin D supplementation on disease outcome. The study also indicates that domesticated cats with spontaneous illnesses may provide a valuable alternative to rodent models in which the effects of vitamin D on health outcomes can be probed without the need to induce disease in otherwise healthy animals.

CHAPTER 3

Investigation of the relationship between vitamin D status and clinical outcomes in dogs with chronic enteropathies

3.1 Introduction

Chronic enteropathies in dogs are a major cause of morbidity and mortality. A diagnosis of a chronic enteropathy (CE) is made in dogs with a chronic history of gastrointestinal signs such as weight loss, vomiting and diarrhoea. Gastrointestinal biopsies from these dogs reveals the presence of an inflammatory infiltrate, for which no underlying aetiology is found following a full diagnostic evaluation [218]. The pathogenesis of canine CE is considered to be multifactorial and includes factors such as abnormal mucosal immunity, disrupted epithelial barrier function, altered intestinal microbial flora, environmental variables and genetic factors [218, 219]. Inflammatory bowel diseases in man, most notably Crohn's disease and ulcerative colitis are thought to be caused by similar factors [220].

It has previously been shown that dogs with a CE have lower serum concentrations of 25(OH)D than healthy dogs and hospitalised dogs with non-gastrointestinal illnesses [5, 6]. In addition, it has also been demonstrated that the severity of the clinical signs, as assessed by the canine inflammatory bowel activity index (CIBDAI), correlates with serum 25(OH)D concentrations in dogs with a CE [5]. However, the prognostic significance of serum 25(OH)D concentrations has not been investigated in dogs with a CE.

In contrast, the relationship between vitamin D status and IBD has been extensively explored in human medicine. In human patients with IBD, vitamin D insufficiency or

deficiency is a frequent finding [221-224]. Higher predicted vitamin D status has been associated with a reduced risk of developing Crohn's disease [225]. Amongst people with IBD, 25(OH)D concentrations are related to disease severity scores and patient quality of life scores [226-228]. Low plasma 25(OH)D concentrations have also been associated with an increased risk of surgery and hospitalisation in patients with either Crohn's disease or ulcerative colitis [229]. Additionally, normalization of 25(OH)D concentrations in patients with Crohn's disease has been associated with a reduction in the risks associated with Crohn's related surgery [229]. Vitamin D status has also been linked to IBD treatment outcomes, as low pre-treatment vitamin D status has been associated with reduced durability of response to anti-tumour necrosis factor (TNF- α) therapy [230]. Studies have also demonstrated improvements in disease activity scores and quality of life scores in Crohn's disease patients supplemented with oral vitamin D [231, 232].

Hypothesis

The hypothesis of this study was that the vitamin D status would not be different in dogs that died or were euthanised due to complications associated with their CE compared to dogs which were alive at follow up or had died due to diseases unrelated to a CE.

Objectives

The aims of this study were to;

- Measure serum concentrations of 25(OH)D, alongside CIBDAI scores, serum albumin concentrations and total serum calcium concentrations in dogs with a confirmed diagnosis of CE and known clinical outcome.

- To assess the effect of serum 25(OH)D concentrations and age, CIBDAI scores, albumin and total calcium concentrations on outcome in dogs with a CE and to assess the possible prognostic significance of serum vitamin D concentration in dogs with a CE.

3.2 Material and Methods

The records of dogs referred to the Hospital for Small Animals, Royal Dick School of Veterinary Studies for investigation of chronic gastrointestinal disease (more than three weeks in duration) between 2007 and 2013 were retrospectively reviewed. Dogs were considered eligible for inclusion in the study if they had presenting clinical signs consistent with a CE which included any of the following: vomiting, diarrhoea, increased borborygmi, abdominal pain, increased or decreased appetite and weight loss. All dogs were considered eligible if they had histopathological evidence of inflammation within the small or large intestines and if there were no clinically relevant abnormalities detected on haematology, biochemistry or abdominal ultrasonography, which were not attributable to CE. In addition, a stored frozen serum sample from each dog collected at the time of diagnosis was required for retrospective analysis of 25(OH)D concentrations. Haematology variables (Total white blood cell count, mature neutrophils, band neutrophils, lymphocytes, monocytes, eosinophils, basophils, total red blood cell counts, packed cell volume, haemoglobin, mean cell volume, mean cell haemoglobin concentration and platelet number) were measured on ADVIA(r) 2120i System with Autoslide (Siemens Medical Solutions Diagnostics Ltd California, USA). Biochemistry variables (albumin, ALT, ALP, bile acids, bilirubin, total calcium, creatinine, globulin, phosphate, potassium, sodium, chloride, urea and glucose) were measured on an ILab650 biochemistry analyser, (Diamond Diagnostics, USA). Clinical records were also reviewed for the results of faecal parasitology. Canine inflammatory

bowel disease activity index scores were calculated as follows: appetite, activity levels, vomiting, faecal consistency, faecal frequency and weight loss were each scored from 0-3. A score of 0 indicated no changes were present, a score of one indicated mild changes, 2 moderate changes and 3 severe changes. In addition a score of one point was added if the faeces contained blood or mucus. The maximum possible score was 20. The area of intestinal tract which was biopsied (duodenum or duodenum and colon) was determined based on presenting clinical signs and was at the discretion of the primary clinician managing the case. A diagnosis of CE was made if there was histological evidence of intestinal inflammation and no clear underlying cause. Dogs were classified as survivors if they were alive at the time of follow up or if they had died from diseases unrelated to their CE. Dogs were classified as non-survivors if they had died or were euthanised as a consequence of their CE.

Vitamin D analysis

Serum samples retained for 25(OH)D measurement were frozen after being used for routine biochemical analysis and were stored at -70°C before being sent to the laboratory for analysis on dry ice. Serum 25(OH)D has been shown to be stable under these conditions [233]. Serum concentrations of 25(OH)D were measured as previously described in detail [234, 235]. Samples were extracted using acetonitrile and applied to C18 Silica Sep-paks. Separation of metabolites was by straight phase high performance liquid chromatography (HPLC) (Waters Associates, Milford, MA, USA) using a Hewlett-Packard Zorbax-Sil Column (Hichrom, Reading, UK) eluted with hexane:propan-2-ol:methanol (92:4:4). Serum 25(OH)D₂ and 25(OH)D₃ were measured separately by application to a second Zorbax-Sil Column eluted with hexane:propan-2-ol (98:2) and quantified by ultraviolet absorbance at 265nm and corrected for recovery (sensitivity 5nmol/L, intra- and inter-assay coefficients of variation 3.0% and 4.2%, respectively) [236]. Total 25(OH)D was defined as the sum of

25(OH)D₂ and 25(OH)D₃. This laboratory is accredited by CPA UK (CPA number 0865) and has been certified as proficient by the international vitamin D Quality Assurance Scheme (DEQAS).

Histopathology

Where available, the slides of the original duodenal biopsies were reviewed by a single veterinary pathologist. Some samples could not be retrieved from archived stores. A qualitative scoring system (WSAVA Standards for the Diagnosis of Gastrointestinal Inflammation in Endoscopic Biopsy Samples) [237], was used to assess the degree of inflammation present. The template for this system assesses the following histological changes: (villous stunting, epithelial injury, crypt distension, lacteal dilatation, and mucosal fibrosis) and inflammatory infiltrates (intraepithelial lymphocytes, lamina propria lymphocytes and plasma cells, lamina propria eosinophils, and lamina propria neutrophils). The changes for each of the variables listed were graded as normal (0), mild (1), moderate (2) or severe (3). The sums of all these variables were added together to determine an intestine inflammatory score which ranged from 0 (normal) to 30 (very severe).

Outcome

For each dog enrolled, follow up data was obtained by reviewing clinical records and by telephone contact with referring veterinary surgeons and owners. Dogs were recorded as survivors if they were alive at follow up or had died due to a non CE related illnesses or non-survivors if dogs had died or were euthanized due to complications associated with CE.

Statistical analysis

Univariable measures were compared between dogs which survived and non-surviving dogs using a Mann-Whitney U test. A Fisher's exact test was used to compare the sex of surviving and non-surviving dogs. A binary logistic regression model was used to estimate the association between outcome (survivors versus non-survivors) and serum 25(OH)D concentrations conditional on a range of other candidate predictors. Stepwise selection of variables was used to minimise Akaike Information Criteria (AIC) which is a parameter penalized measure of model fit. Intestine inflammatory scores were classified into three approximate tertiles (low <7, medium 7-8, high>8) for the regression model. The statistical analysis was performed using R statistical system (R Development Core Team (2012)).

Ethical Review

Informed consent for the use of residual clinical blood samples for research purposes was obtained at admission for each dog enrolled. Ethical approval for the study was obtained from the University of Edinburgh's Veterinary Ethical Review Committee.

3.3 Results

Signalment

Forty-one dogs were included in the study. There were 15 non-survivors and 26 survivors. In the non-survivors group, 2 dogs were intact males, 7 were neutered males, 1 was an intact female and 5 were neutered females. In the survivors group, 7 dogs were intact males, 11 were neutered males and 8 were neutered females. Breeds in the non-survivor groups included Border Collie (n=1), Boxer (n=3), Cavalier King Charles Spaniel (n=1), Cross Breed (n=3), German Short Haired Pointer (n=1), Hungarian Vizsla (n=1), Italian

Greyhound (n=1), Pyrenees Mountain Dog (n=1), Springer Spaniel (n=1), Staffordshire Bull Terrier (n=1) and West Highland White Terrier (n=1). In the survivors groups breeds included Border Terrier (n=1), Boxer (n=7), Cocker Spaniel (n=1), Cavalier King Charles Spaniel (n=1), Chinese Crested (n=1), Cross Breed (n=1), Irish Setter (n=2), Labrador Retriever (n=2), Lurcher (n=2), Rottweiler (n=1), Shar pei (n=1), Shetland Sheep Dog (n=1), Springer Spaniel (n=1), Staffordshire Bull Terrier (n=1), Toy Poodle (n=2) and Yorkshire Terrier (n=1).

Clinical Findings

The median duration of clinical signs at diagnosis was 3 months in the non-survivors (range 1 month-10 months) and 3.5 months (range 0.75 months-24 months) in the survivors group. Haematology, biochemistry and abdominal ultrasonography findings did not reveal any clinically relevant abnormalities in any of the 41 dogs which could not be attributed to their CE. Faecal parasitology was performed in 36 dogs and did not reveal evidence of parasitic infection in any of the samples. Twelve dogs underwent gastroduodenoscopy and 29 dogs had both gastroduodenoscopy and colonoscopy. Histopathological examination of duodenal biopsies in the non-survivors revealed lymphoplasmacytic enteritis (5) and mixed lymphoplasmacytic and eosinophilic enteritis (10). In the survivor group histopathological diagnosis based on duodenal biopsies included lymphoplasmacytic enteritis (8) and mixed lymphoplasmacytic and eosinophilic enteritis (18). Follow up data for the survivor group ranged from 18-75 months (median 27 months). In the non-survivors group the follow up data was available until death occurred. This ranged from 4 days to 24 months (median 2 months).

Outcome

Fifteen dogs died or were euthanized as a result of their CE (table 1). The age of the dogs at presentation which subsequently died or were euthanised due to their chronic enteropathy ranged from 9 to 114 months (median 96 months). Dogs which subsequently died had been treated with dietary changes and antibiotics (n=2), dietary changes, prednisolone and antibiotics (n=7) and dietary changes, antibiotics, prednisolone and other immunosuppressive medications (n=6). Twenty six dogs did not die as a result of gastrointestinal disease (table 1). Sixteen dogs were alive at follow up and ten had died due to non-gastrointestinal diseases. The age of the dogs in the survivors group ranged from 6 to 96 months (median 60.5 months). Dogs in this group were treated with dietary changes and antibiotics (n=10), dietary changes and prednisolone (n=1), dietary changes, prednisolone and antibiotics (n=3), dietary changes, prednisolone, antibiotics and other immunosuppressive drugs (n=1), diet alone (n=10) and gastroprotectants (n=1).

Univariable analysis revealed that serum 25(OH)D, albumin and total calcium concentrations were significantly lower in non-survivors compared to survivors (table 1, fig. 1). Age and CIBDAI scores were significantly higher in non-survivors compared to survivors (table 1). There was no significant difference in the number of male and female dogs between the two groups (table 1).

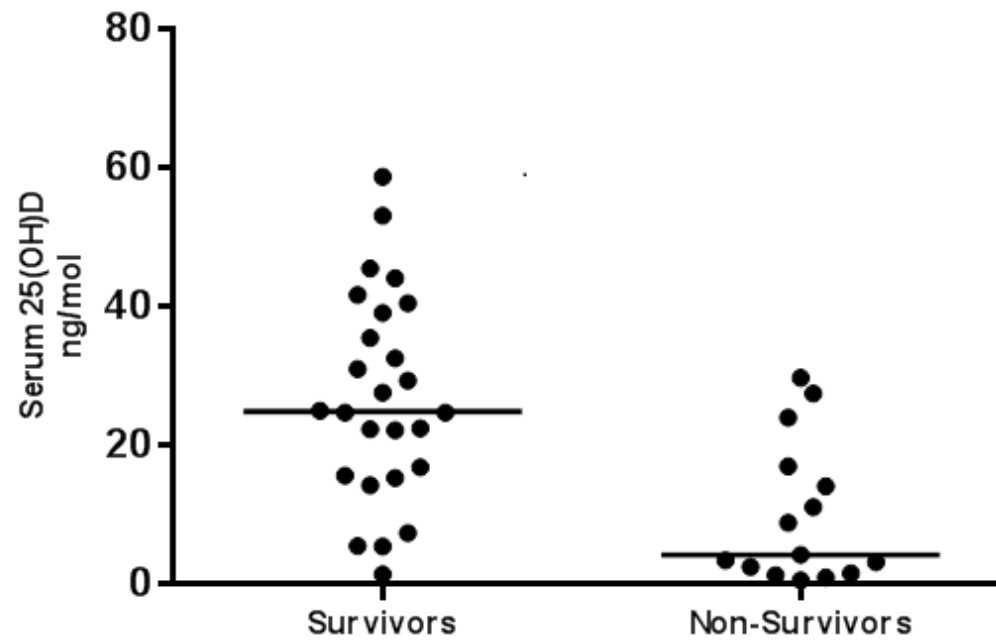


Figure 1: Serum 25(OH)D concentrations in surviving and non-surviving dogs with a CE.

	Survivors (n=26)	Non-survivors (n=15)	p value
Age (months)	60.5 (33.0)	96.0 (48)	0.004*
Calcium mmol/l	2.42 (0.26)	2.06 (0.57)	0.002*
Albumin g/l	33.35 (7.95)	24.50 (13.9)	0.0007*
CIBDAI	6 (3)	10 (5)	0.0022*
25(OH)D ng/ml	24.90 (22.20)	4.3 (15.4)	0.0003*
Male/female	18/8	9/6	0.733

Table 1: Univariable analysis of clinical and biochemical variables in surviving and non-surviving dogs. The data shows the median value for each variable and the interquartile range. * denotes statistical significance $P < 0.05$

A binary logistic regression model was performed to estimate the association between outcome and serum 25(OH)D concentrations conditional on a range of other candidate predictors. CIBDAI was not included in this model since it has previously been shown that this measure strongly correlates with serum 25(OH)D concentrations [5]. In this analysis intestinal inflammation scores were also included. These scores were available for 34 of the 41 dogs. There was no significant difference in histopathology scores between dogs which died compared to ones which survived ($p=0.06$). There were 10 dogs with a score of less than 7, 10 dogs with a score of 7 or 8 and 14 dogs with a score of 9 or greater. The initial model included age, sex, histopathology score as a tertile, serum calcium, albumin and 25(OH)D concentrations. After stepwise AIC selection, the optimal predictive final model

used serum 25(OH)D concentrations and age demonstrating that vitamin D status was an independent predictor of mortality in dogs with CE (table 2). Table 3 shows the consequences on model fit following the re-introduction of discarded predictors, demonstrating that the model based on 25(OH)D concentrations and age was optimal.

	Odds ratio (95% CI)
25(OH)D ng/ml	1.08 (1.02-1.18)
Age months	0.97 (0.93-1.00)

Table 2: Results from the final binary logistic regression model of survivors versus non-survivors

Added predictor	AIC
(Final model which includes 25(OH)D and age)	36.97
Albumin	38.97
Calcium	38.81
Sex	38.60
Histology tertile	40.31

Table 3: Impact on model AIC following addition of dropped predictors. An increase in AIC represents a poorer parameter penalized model fit.

3.4 Discussion

The main finding of this study is that serum 25(OH)D concentrations were significantly lower at time of diagnosis in dogs which died or were euthanised as a result of a CE. This is an important finding since it is presently difficult to predict outcomes in dogs with a CE. Previous studies have shown that older age, hypoalbuminaemia and higher CIBDAI scores are predictive of a poorer outcome in dogs with a CE, findings which are further supported by this research [238-240]. This study, which demonstrates that serum 25(OH)D concentration at the point of diagnosis is a prognostic marker in dogs with a CE, will hopefully help provide additional valuable prognostic information to owners and veterinary surgeons managing dogs with a CE. Further work is needed to determine the optimal cut-off value for vitamin D as a prognostic marker.

The mechanism(s) underlying a hypovitaminosis D state in canine CE is unknown. Reduced dietary intake of vitamin D in dogs with a CE could be an important cause of hypovitaminosis D, especially as dogs do not cutaneously produce vitamin D [16]. As low vitamin D status in dogs with a CE has been associated with a decreased appetite [5], this may, in part, explain the reduced serum 25(OH)D concentrations that are frequently observed in dogs with a CE. However, Gow et al have also found that dogs with a CE had lower 25(OH)D concentrations than hospitalised ill dogs with non-gastrointestinal illnesses, and many of these dogs also had reduced appetites [5]. Consequently, the influence of appetite on vitamin D status in dogs with a CE remains unclear.

Circulating 25(OH)D is bound to vitamin D binding protein and albumin [241]. Enteric loss of albumin is regarded to be a significant problem in many dogs with a CE, notably in cases of protein losing enteropathies [242]. Consequently, loss of protein-bound vitamin D into the

gastrointestinal tract may account for the low vitamin D status in some dogs with a CE. This may explain previous work which has demonstrated a correlation between serum albumin concentrations and vitamin D status in dogs with a CE [5]. Similarly, albumin concentrations have also been shown to be a predictor of serum 25(OH)D concentration in humans and cats with IBD [10, 243]. Albumin can also decrease in inflammatory diseases as part of the acute phase response [244]. Studies have shown the systemic inflammatory response may be a confounding factor in determining vitamin D status [245]. This is supported by investigations which have shown that serum vitamin D binding proteins decreases in the face of acute inflammation and urinary excretion of vitamin D binding protein increases in the face of inflammation [148, 246]. Therefore, a reduction or loss of vitamin D from the plasma compartment due to redistribution of its binding proteins (serum albumin and vitamin D binding protein) may account for the decreases seen in 25(OH)D concentrations in acute inflammation and inflammatory diseases.

Malabsorption may also contribute to low vitamin D status in human patients with Crohn's disease and this could potentially influence serum 25(OH)D concentrations in dogs with a CE. However, whilst vitamin D absorption appears to be reduced in patients with Crohn's disease compared to healthy controls, there seems to be substantial variation in absorption of vitamin D in these patients [247]. Further studies are needed in both people and dogs with CE to clarify the potential role of vitamin D malabsorption in driving a hypovitaminosis D state.

Although hypovitaminosis D in CE has traditionally been considered to be a result of intestinal disease, there is growing evidence that hypovitaminosis D may contribute to the initiation of intestinal inflammation. Supporting evidence for a link between

hypovitaminosis D and CE comes from rodent models which have demonstrated that vitamin D receptor knock out ($VDR^{-/-}$) mice are more susceptible to experimental forms of inflammatory bowel disease. For example, experimentally induced colitis in $VDR^{-/-}$ mice was significantly more severe compared to wild-type mice with chemically induced colitis [64, 248]. Furthermore, it has also been demonstrated that supplementing wild type mice with vitamin D can decrease the severity of gastrointestinal inflammation with chemically induced colitis [248]. Additionally, it has been shown that feeding mice a vitamin D restricted diet can predispose to IBD [249].

Vitamin D has also been shown to profoundly modulate pro-inflammatory responses and to promote immunotolerance [250, 251]. Therefore, lack of vitamin D may drive abnormal inflammatory and immune processes. Subnormal vitamin D concentrations therefore may contribute to the inflammation seen in people with IBD and dogs with CE. Loss of tolerance to normally harmless bacterial and dietary antigens is hypothesised to be important for the development of canine CE [219]. Vitamin D may be important in regulating the immune response to commensal gut flora and maintaining normal bacterial populations. For example, dysbiosis was also reported in $VDR^{-/-}$ mice and mice $Cyp27B1^{-/-}$ mice [252] and dysbiosis may contribute to CE in dogs [253]. Therefore, the effects of vitamin D on the intestinal microbiota may also account for the association between serum 25(OH)D and inflammatory bowel disease.

There is growing evidence linking vitamin D deficiency with disrupted intestinal mucosal barrier function. It has been proposed that altered epithelial barrier function, resulting in increased epithelial permeability, leads to increased exposure of the mucosal immune system to luminal antigens and that this may contribute to initiation and perpetuation of chronic

inflammation [254]. This hypothesis is supported by the observation that intestinal permeability is increased in people with naturally-occurring inflammatory bowel disease and their unaffected relatives [255]. Similar findings have been reported in dogs with naturally-occurring CE where increased paracellular permeability has been demonstrated by lactulose to rhamnose absorption tests [256, 257]. *In-vitro* studies have demonstrated that calcitriol can markedly enhance tight junction protein expression [64]. The same study also demonstrated that VDR^{-/-} mice were more susceptible to mucosal injury than wild type mice.

A feature of inflammatory bowel disease is excessive intestinal cell apoptosis [258, 259]. It has recently been demonstrated that intestinal epithelial vitamin D receptor signalling inhibits experimental colitis and targeted expression of VDR in intestinal epithelial cells of mice protected them from experimental forms of colitis [260]. These results demonstrate that gut epithelial VDR signalling inhibits colitis by protecting the mucosal epithelial barrier. The same group demonstrated that reconstitution of VDR^{-/-} intestinal epithelial cells with *VDR* transgenes protected VDR^{-/-} mice from severe colitis and death by reducing intestinal epithelial cell apoptosis [260]. As these VDR^{-/-} mice have a hyper-responsive immune system, the results indicated that intestinal VDR signalling has protective effects in IBD. Treating mice with chemically induced colitis with vitamin D analogues also appears to reduce intestinal epithelial cell apoptosis [261]. Therefore, vitamin D status may be associated with IBD in man and CE in dogs due to its effects on altered barrier function.

Culture of colon cancer cells with tumour necrosis factor α (TNF- α) has been shown to suppress VDR and concurrently upregulate microRNA-346 (miR-346). In turn miR-346 inhibits VDR expression [262]. These results indicate that during mucosal inflammation

VDR expression is down-regulated. The loss of mucosal VDR then results in a loss of the mucosal epithelial barrier integrity, further driving mucosal inflammation. Research is required to investigate whether similar changes in VDR expression occur in dogs with CEs. It is speculated that as vitamin D can induce VDR expression [263] and suppresses TNF- α [60, 264], treatment with vitamin D analogues may help prevent intestinal epithelial cell apoptosis and ameliorate the clinical signs associated with inflammatory bowel disease [264]. A reduction in intestinal epithelial cell VDR expression has been reported in naturally occurring IBD [260, 261].

It is currently unclear if supplementing patients diagnosed with IBD with vitamin D improves patient outcomes. A recent meta-analysis of animal and human trials concluded that whilst many studies reported an improvement in IBD related symptoms or pathology after vitamin D supplementation, there is insufficient evidence to currently recommend vitamin D treatment for human cases of inflammatory bowel disease [265]. Therefore further research is needed into the potential role of vitamin D in the management of these diseases. In addition there is evidence to suggest that the hypovitaminosis D seen in people with inflammatory bowel disease will correct when the disease is managed independent of supplementation with exogenous vitamin D, suggesting that low vitamin D concentrations in IBD may be a consequence rather than the cause of IBD [266]. Studies are required in dogs to assess if supplementation with vitamin D or vitamin D analogues is beneficial in dogs with a CE.

There are some limitations in this study. Firstly, there was no standardisation in treatment regimens which the dogs received before or after diagnosis of CE. This is a result of the retrospective nature of the study design. Some medications can influence vitamin D

metabolism in humans. However, it is unclear whether many commonly used drugs can influence vitamin D metabolism in dogs. For example, it has previously been shown that glucocorticoids do not alter vitamin D metabolism in dogs [182]. In light of paucity of information on how drugs influence vitamin D homeostasis it was elected to take an unbiased approach of not excluding any cases based on previous medical therapy. Additionally, differences in treatments may have affected outcomes in the dogs in this study independently of vitamin D status. Another limitation of this study was that the results of faecal parasitology were missing from 5 of the dogs recruited for this study. Undetected parasite infections may have influenced the results of the variables measured for this study. However, it is unlikely that this has a major impact on test results as within this hospital population dogs are almost invariably prophylactically treated with appropriate worming medication especially prior to referral for investigation of gastrointestinal disorders. The observational nature of the study is another limitation, as it prevents conclusions being drawn as to whether low serum vitamin D concentrations are a cause rather than a consequence of vitamin D deficiency.

3.5 Conclusion

In summary, this study demonstrates that serum 25(OH)D concentrations are predictive of clinical outcome in dogs with CE. Although, causality cannot be inferred from these results, the finding that low serum 25(OH)D concentrations are negatively correlated with outcome highlights the need to further examine the relationship between vitamin D homeostasis and the risk of developing a CE and the effect of vitamin D status on outcome in dogs with a CE. The results of this study support the initiation of randomised placebo controlled studies utilising vitamin D supplementation in order to address these questions.

CHAPTER 4

Vitamin D status and markers of inflammation in dogs with chronic enteropathies

4.1 Background

There is a growing body of evidence that vitamin D status is negatively associated with markers of inflammation (including circulating pro-inflammatory cytokines and acute phase proteins) in a number of diseases including obesity [267, 268], inflammatory polyarthritis [269], diabetes mellitus [270], autoimmune diseases [66, 271], inflammatory bowel disease [272, 273] and human immunodeficiency virus [274]. Furthermore, low vitamin D status has been associated with increased markers of inflammation in healthy humans [275-277].

The reasons why low vitamin D status is negatively associated with inflammation are unclear, but are likely to be related to the role vitamin D plays in influencing immune responses. The vitamin D receptor (VDR) is found on most types of immune cells including: macrophages, dendritic cells, T-lymphocytes and B-lymphocytes [189]. Vitamin D can reduce inflammation by inhibiting the proliferation of immune effector cells and the production of inflammatory cytokines while enhancing production of anti-inflammatory cytokines [42]. Vitamin D can also act promote immunotolerance by increase regulatory T-cell populations [278, 279]. Despite the anti-inflammatory functions of vitamin D, vitamin D is also known to enhance the innate immune response to bacteria, by increasing production of anti-microbial peptides such as cathelicidin [57-59].

The role of vitamin D in modulating inflammatory responses has been investigated by studying the effects of vitamin D supplementation on markers of inflammation in people. In healthy adults with low serum 25(OH)D concentrations, vitamin D supplementation has

been shown to increase concentrations of both circulating pro and anti-inflammatory cytokines [280]. In ill patients, vitamin D administration appears to reduce some markers of inflammation in patients with early chronic kidney disease [281], end-stage renal disease [282], congestive heart failure [283], systemic lupus [284] and colorectal adenoma [285]. In addition markers of inflammation decreased in elderly women with vitamin D insufficiency in response to supplementation with a mega-dose of vitamin D3 [286]. However, it is important to bear in mind that many other studies investigating the effects of vitamin D supplementation on markers of inflammation have failed to demonstrate *in vivo* anti-inflammatory effects. For example, high dose vitamin D treatment appears not to result in a decrease in inflammatory markers in people with low vitamin D status diagnosed with: pre-diabetes and type 1 diabetes [270, 287], hypertension [288] and urticaria [289]. Furthermore, increases in pro-inflammatory cytokines were documented in patients with osteoporosis after vitamin D supplements were administered [290]. There are many potential explanations for why the results of different studies reported differing effects of vitamin D supplementation on markers of inflammation. These include: differences in the duration for which vitamin D supplementation was given, the type of vitamin D used for supplementation and the effects of various diseases. In addition, observational studies have demonstrated an U-shaped association between 25(OH)D and CRP concentrations, which indicates that higher 25(OH)D concentrations may also be related to pro-inflammatory states [291, 292]. Benefits of vitamin D supplementation may therefore only be seen in patients with overt vitamin D deficiency and excessive supplementation may be detrimental.

The relationship between vitamin D status and inflammation is poorly understood in dogs. Serum 25(OH)D concentrations are commonly reduced in a number of inflammatory diseases in dogs including congestive heart failure [3], spirocercosis [4], protein losing enteropathy [5, 6] and renal disease [7]. Low vitamin D status has been negatively associated with CRP in dogs with haemoabdomens [8]. However, other studies have

reported a positive association between vitamin D and CRP concentrations in racing sled dog [293]. Further work is needed to address the relationship between vitamin D and inflammation in dogs.

Dogs with CE represent a spontaneous model in which to explore the relationship between vitamin D status and inflammation. It has been previously demonstrated that dogs with a CE can have a range of serum vitamin D concentrations, ranging from profound deficiency to sufficiency [5, 6]. This thesis has also shown in chapter three that serum vitamin D concentrations are predictive of clinical outcomes in dogs with CE, yet the relationship between 25(OH)D concentrations and inflammation in dogs with CE is poorly understood. A number of markers of systemic inflammation, including serum cytokines and leukocyte profiles can be measured in dogs [294]. In addition the WSAVA gastrointestinal histopathology scoring system for endoscopic biopsies [237] provides a standardised means by which the extent of inflammation within gastrointestinal biopsies can be assessed.

Chronic enteropathies are commonly diagnosed in dogs. Like IBD in man, CE represents a range of conditions associated with gastrointestinal inflammation. Again, similar to humans, hypovitaminosis D is a common clinical finding in dogs with CE [5, 6]. Unlike people, dogs do not produce vitamin D cutaneously and most are fed commercial diets containing relatively standard amounts of vitamin D. This limits the number of variables which influence vitamin D status, making dogs an attractive spontaneous disease model to study the relationship between systemic and gastrointestinal inflammation and serum 25(OH)D concentrations. This is of particular interest because although low vitamin D status is commonly reported in patients with IBD [221-224], the relationship between vitamin D status and IBD associated inflammation in human patients is not yet well defined. Investigators have assessed the link between the incidence of sub-optimal vitamin D

concentrations and increased markers of local and systemic inflammation in patients with IBD. The results of these studies are conflicting. For example, changes in markers of systemic inflammation such as CRP, fibrinogen, white blood cell count, platelet count, TNF- α and erythrocyte sedimentation rate appear to be unrelated to vitamin D status in patients with IBD [222, 295, 296]. In contrast, faecal markers of gastrointestinal inflammation have been shown to be increased in patients with hypovitaminosis D and IBD [273, 296]. Furthermore, vitamin D insufficiency has also been associated with reduced concentrations of the anti-inflammatory cytokine IL-10 in patients with ulcerative colitis, suggesting that poor vitamin D status may be linked to reduced anti-inflammatory capacity in this group [295]. Ongoing work is needed to define the role of vitamin D in the initiation and perpetuation of gastrointestinal inflammation. Dogs may provide an attractive model in which this relationship may be studied.

Hypothesis

The study hypothesis was that vitamin D status would negatively correlate with markers of systemic and intestinal inflammation in dogs with CE.

Objectives

The aim of this investigation was to assess the association between serum vitamin D status and inflammation in dogs with CE by measuring 25(OH)D concentrations alongside:

- Whole blood neutrophil counts, monocytes counts, lymphocyte counts and eosinophil counts.
- Pro-inflammatory cytokines, IL-2, IL-6, IL-8 and TNF- α
- Intestinal inflammation scores as a marker of local gastrointestinal inflammation.

The study also aimed to determine if serum 25(OH)D concentrations correlated to a specific ‘inflammatory signature’ that could describe the changes in inflammatory markers seen in dogs with a CE.

4.2 Material and Methods

The records of dogs referred to the Hospital for Small Animals, Royal Dick School of Veterinary Studies for investigation of chronic gastrointestinal disease (more than three weeks in duration) were retrospectively reviewed. Dogs were considered eligible for inclusion in the study if they had presenting clinical signs consistent with a CE which included any of the following: vomiting, diarrhoea, increased borborygmi, abdominal pain, increased or decreased appetite and weight loss. All dogs were considered eligible if they had histopathological evidence of inflammation within the small or large intestines and if there were no clinically significant abnormalities detected on haematology, biochemistry or abdominal ultrasonography. In addition the faeces of all dogs were negative for both helminth and giardia infections. Biochemistry variables were measured on an ILab650 biochemistry analyser, (Diamond Diagnostics, USA). The area of the intestinal tract which was biopsied (duodenum or duodenum and colon) was determined based on presenting clinical signs and by the primary clinician managing the case. A diagnosis of CE was made if there was histological evidence of intestinal inflammation and no clear underlying aetiology was diagnosed. For inclusion, residual stored serum samples were required for retrospective assessment of 25(OH)D status.

Vitamin D analysis

Serum samples retained for 25(OH)D measurement were frozen after being used for routine biochemical analysis. Serum was stored at -70°C before being sent to the laboratory for analysis on dry ice. Serum concentrations of 25(OH)D were measured as previously

described in detail [234, 235]. Samples were extracted using acetonitrile and applied to C18 Silica Sep-paks. Separation of metabolites was by straight phase high performance liquid chromatography (HPLC) (Waters Associates, Milford, MA, USA) using a Hewlett-Packard Zorbax-Sil Column (Hichrom, Reading, UK) eluted with hexane:propan-2-ol:methanol (92:4:4). Serum 25(OH)D₂ and 25(OH)D₃ were measured separately by application to a second Zorbax-Sil Column eluted with hexane:propan-2-ol (98:2) and quantified by ultraviolet absorbance at 265 nm and corrected for recovery (sensitivity 5nmol/L, intra- and inter-assay coefficients of variation 3.0% and 4.2%, respectively) [236]. This Supraregional Assay Service laboratory is accredited by CPA UK (CPA number 0865) and has been certified as proficient by the international vitamin D Quality Assurance Scheme (DEQAS). Total 25(OH)D is defined as the sum of 25(OH)D₂ and 25(OH)D₃.

Cytokine analysis

Canine pro-inflammatory cytokines (IL-2, IL-6, IL-8 and TNF- α) were measured from serum samples using a multiplex electrochemiluminescence immunoassay system (Meso Scale Discovery; MSD) as previously described [294]. Assay diluent (25 μ L) was added to all wells, plates sealed and incubated for 30 minutes at room temperature on an orbital shaker (600 rpm). Samples and standards, diluted in assay diluent, were added at 25 μ L per well. Plates were again sealed and incubated for a further 2 hours at room temperature with shaking. At the end of the incubation period, wells were washed three times with 200 μ L/well phosphate-buffered saline (PBS), supplemented with 0.05% Tween 20 (Sigma–Aldrich) for 30 seconds, and then discarded. Detection antibody was added at 25 μ L per well, plates sealed and incubated for a further 1 hour at room temperature with shaking. Plates were washed three times and 150 μ L of MSD Read Buffer added to each well, then electrochemiluminescence was measured using the MSD Sector Imager 2400 plate reader.

Histopathology

Where available, slides of the original duodenal biopsies were reviewed by a single veterinary pathologist. A qualitative scoring system (WSAVA Standards for the Diagnosis of Gastrointestinal Inflammation in Endoscopic Biopsy Samples) [237] was used to assess the degree of inflammation. The template for this system assesses the following histological changes: (villous stunting, epithelial injury, crypt distension, lacteal dilatation, and mucosal fibrosis) and inflammatory infiltrates (intraepithelial lymphocytes, lamina propria lymphocytes and plasma cells, lamina propria eosinophils, and lamina propria neutrophils). The changes were graded as normal (0), mild (1), moderate (2) or severe (3). The sums of all these parameters were added together to determine an intestine inflammatory score which ranged from 0 (normal) to 30 (very severe).

Statistical Analysis

Initially statistical analysis was performed using GraphPad Prism version 6 (GraphPad software, La Jolla, CA). A Spearman's rank coefficient was used to investigate potential correlations between serum 25(OH)D concentrations and intestinal inflammation scores, total white blood cell count, neutrophil, lymphocyte, monocyte and eosinophil concentrations and serum cytokines. A P value of <0.05 was considered to be statically significant. In order to investigate the relationship between multiple inflammatory markers and serum 25(OH)D concentrations, individual variables were examined using histograms and parameters which were not normally distributed (Anderson-Darling test) were transformed. Log10 transformation was undertaken except for square root transformation of eosinophil numbers since some eosinophil numbers were zero. The relationship between age, sex and 25(OH)D was examined using scatter plots and a linear regression model. The relationship between 25(OH)D and inflammatory markers was investigated using linear regression models. Age and sex were included in initial models in order to examine for any

confounding effects. Final models were selected by removing potential confounders if their removal did not worsen model fit as assessed by the Akaike information criterion (AIC, a penalty parameter penalised measure of model fit). As one of the study aims was to examine if 25(OH)D concentrations correlated to an inflammatory signature, medoid based partitioning was used to assign haematology parameters to three clusters. Three clusters was chosen on the basis of a consensus of results from 30 cluster count algorithms [297]. These clusters were identified independently of 25(OH)D concentrations. A Kruskal Wallis test was used to determine if there was a difference in 25(OH)D concentrations between the three clusters [298]. Tukey and Kramer posthoc test for pairwise comparisons were then used to identify which clusters differed. Data was collected using Excel and analysed using R statistical software system [299]. A p value of <0.05 was used to define statistical significance.

4.3 Results

Thirty-nine dogs with a diagnosis of a CE were enrolled. Breeds enrolled included; Boxer (9), cross breed (5), Caviller King Charles Spaniel (2), Labrador (2), Lurcher (2), Springer Spaniel (2), Staffordshire Bull Terrier (2) and one of each of the following breeds: Border Collie, Border Terrier, Cocker Spaniel, Douge De Bordeaux, German Short Haired Pointer, Greyhound, Hungarian Vizsla, Irish Setter, Italian Greyhound, Pyrenees Mountain Dog, Shar pei, Shetland Sheep dog, Toy poodle, Yorkshire Terrier and a West Highland White Terrier. The age range of dogs included was 6 to 136 months (median 65 months).

Haematology, biochemistry, faecal parasitology and abdominal ultrasonography did not reveal any significant clinical abnormalities in any of the 39 dogs that was not attributed to CE. Sixteen dogs underwent upper endoscopy and 23 dogs had both upper and lower endoscopy. Thirty-two dogs had duodenal biopsy samples which were available for histological review by a veterinary pathologist who was blinded to the clinical history,

cytokine results and vitamin D status of the dogs. Other samples could not be retrieved from achieved stores. Cytokine analysis was available in 23 cases.

Age and sex were not predictive of serum 25(OH)D concentrations in a linear regression model. The relationships between serum 25(OH)D concentrations and haematology, intestinal inflammation score and serum cytokine results are shown in figure 1. Numbers of neutrophils and monocytes, intestinal inflammation score and serum IL-2 and IL-8 concentrations were negatively associated with 25(OH)D concentrations (table 1). Three clusters were identified in the haematology data (figures 2 and 3). There was a significant difference in 25(OH)D concentrations between the three clusters (p=0.009). Post-test analysis revealed a significant difference between clusters 2 and 3 (p=0.006).

Variable	Co-efficient (SE)	P value
Neutrophil number (log10)	-0.011 (0.0026)	0.0001 *
Monocyte number (log10)	-0.016 (0.0039)	0.0002 *
Eosinophil number (sqrt)	0.008 (0.0044)	0.092
Lymphocyte number (log10)	0.005 (0.0035)	0.156
Duodenal histopathology score (log10)	-0.012 (0.0041)	0.006 *
IL-2 (log10)	-0.015 (0.0074)	0.048 *
IL-6 (log10)	-0.011 (0.0062)	0.086
IL-8 (log10)	-0.018 (0.0064)	0.013 *
TNF- α (log10)	-0.014 (0.0070)	0.061

Table 1: Results of logistic regression model of inflammatory parameters on serum 25(OH)D concentrations *denotes statistical significance P<0.05

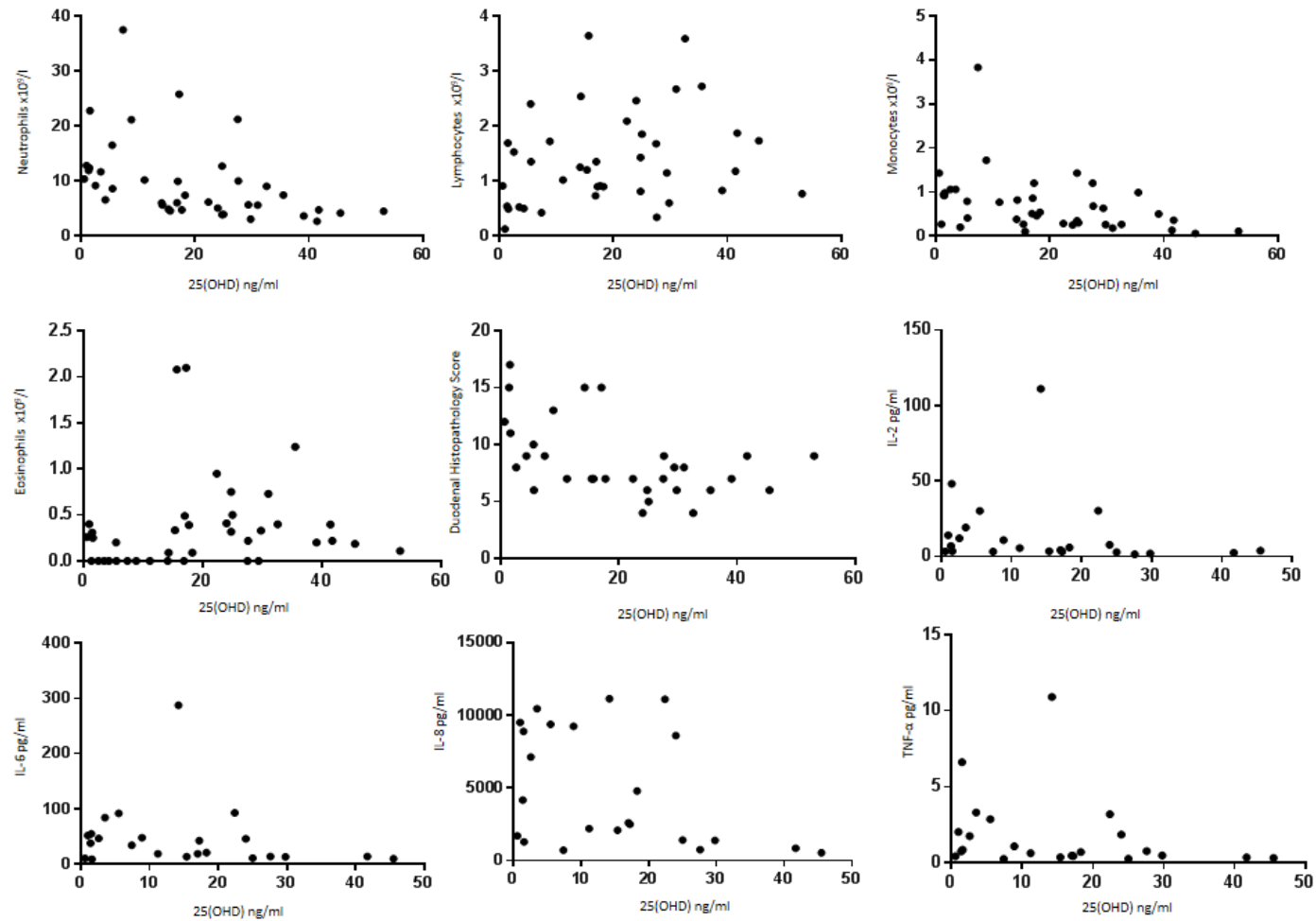


Figure 1: Relationship between haematology, serum cytokines and duodenal inflammatory score and serum 25(OH)D concentrations

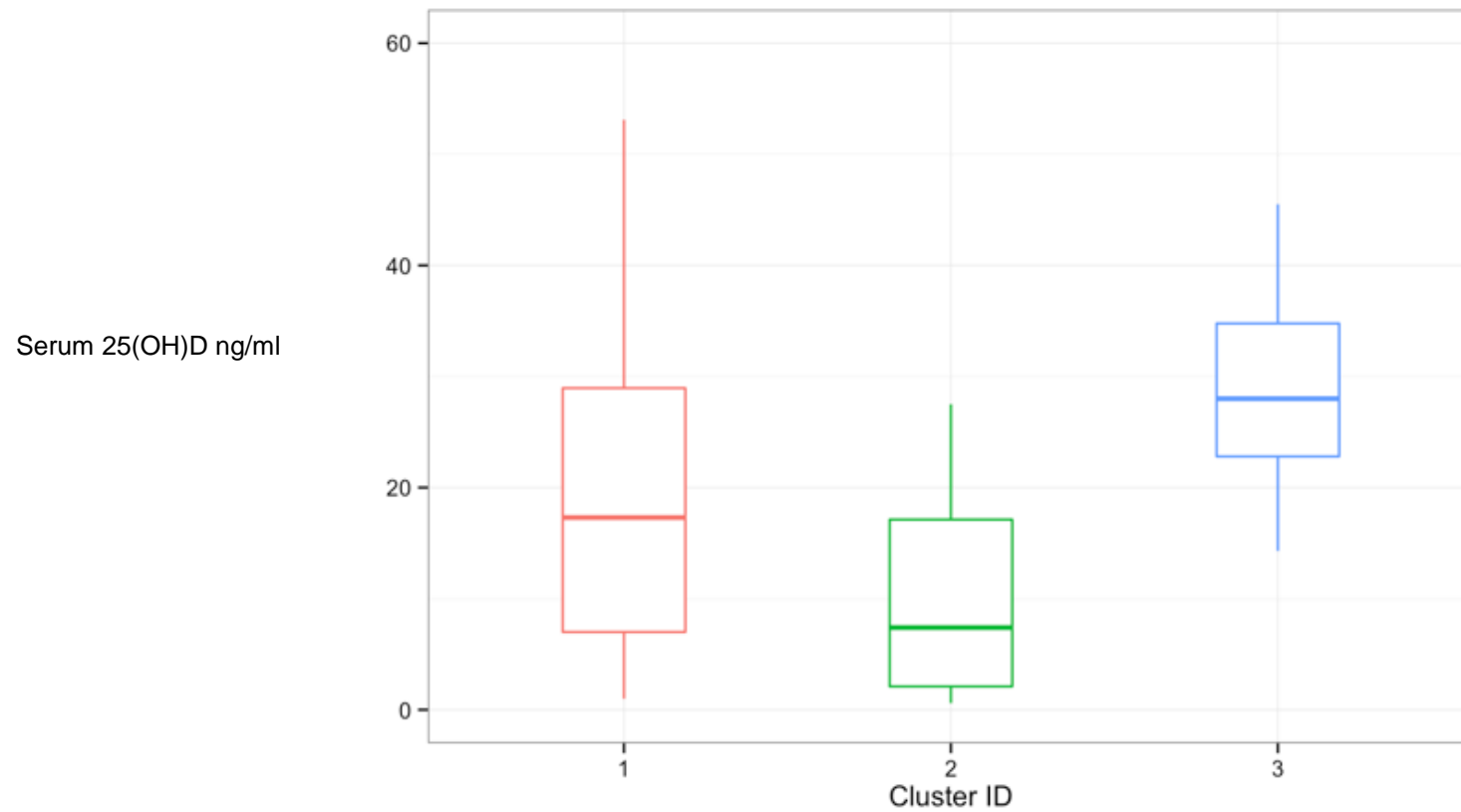


Figure 2: Box and whisker plot of serum 25(OH)D concentrations by cluster. The box extends from 25% to 75% percentile with the median and the whiskers extend to limits of the data.

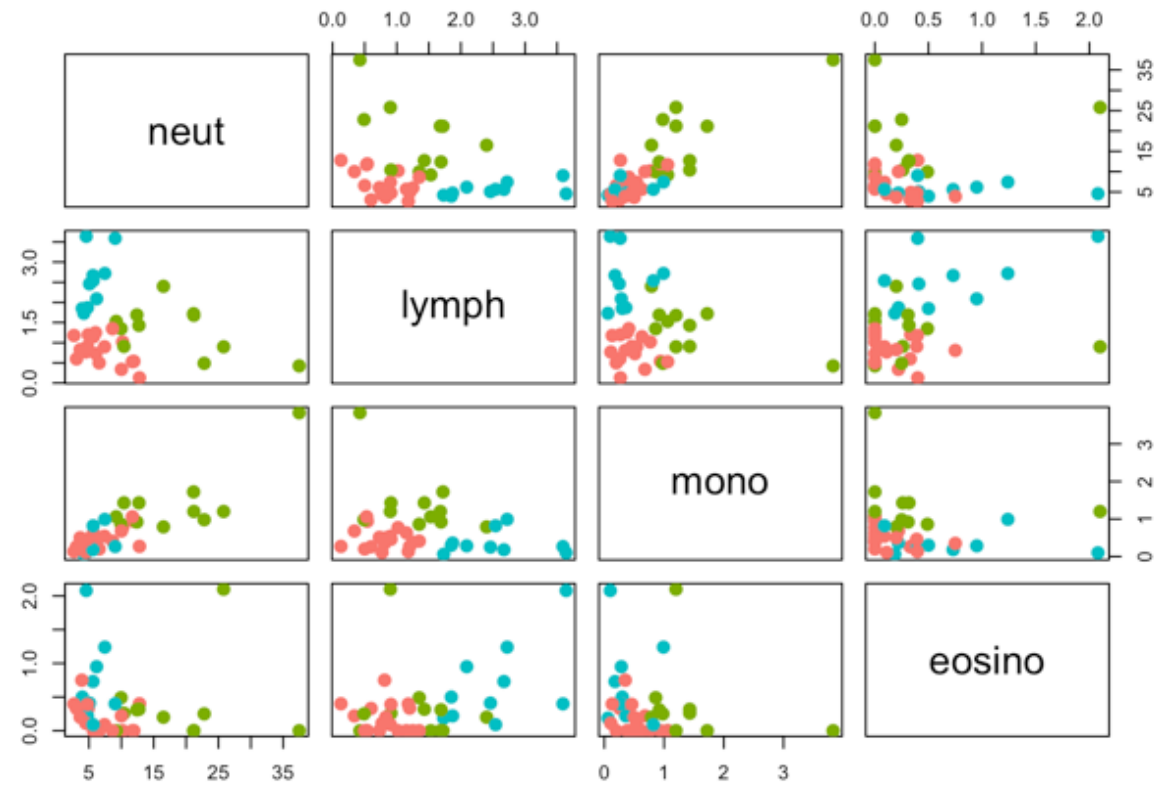


Figure 3: Pair-wise scatter plot of number of neutrophils (neut), monocytes (mono), eosinophils (eosino) and lymphocytes (lymph) labelled by cluster (red cluster 1, green cluster 2, and blue cluster 3). Values on x and y axis denote cell concentrations $\times 10^9/\text{l}$.

4.4 Discussion

The central finding of this study is that serum 25(OH)D concentrations negatively correlate with systemic markers of inflammation in regards to neutrophil and monocyte numbers, serum IL-2 and IL-8 concentrations and local inflammation as measured by duodenal histopathology scores. Linear regression and medoid based partitioning analysis demonstrated that dogs with low vitamin D status had an inflammatory signature consisting of high neutrophil and monocyte numbers and low lymphocyte numbers. A typical stress leukogram in dogs consists of neutrophilia, monocytosis and lymphopenia and similar changes can also be seen with an inflammatory leukogram. These results demonstrate that lower serum vitamin D status is seen associated with increased numbers of neutrophils, monocytosis and lymphopenia. These changes can be seen in a number of inflammatory conditions and are not-specific to dogs with a CE, however they provide evidence of an association between reduced serum 25(OH)D concentrations and systemic inflammation in this population of dogs.

The results of this study also show that IL-2 and IL-8 concentrations are increased in the serum of dogs with CE and lower 25(OH)D concentrations. Cell culture models indicate that the pro-inflammatory cytokine, IL-2 is under direct transcriptional regulation by calcitriol and that calcitriol decreases IL-2 production [300]. Vitamin D supplementation has been associated with decrease in circulating IL-2 concentrations in children with atopy [301]. As seen in the dogs in this study, increased IL-2 concentrations have been reported in vitamin D insufficient adults [302]. Serum IL-8 were also increased in this population of dogs with a CE. In human IBD IL-8 is an important inflammatory mediator, which plays a role in the initiation and maintenance of IBD by recruiting neutrophils into inflamed tissues [303]. Increases in serum IL-8 concentrations have also been reported in human patients with IBD [304]. It is also interesting that vitamin D has been shown to exert anti-inflammatory effects

by decreasing IL-8 production in intestinal cell cultures [305]. The role of these cytokines in canine CE and the effects of vitamin D status on their expression in dogs is worthy of further investigation.

The results of this study indicate that low serum vitamin D concentrations are associated with more severe inflammation as assessed by intestinal inflammation scores. This provides evidence of an association between local inflammation and reduced vitamin D concentrations. Although faecal markers of local gastrointestinal inflammation have been associated with low vitamin D status in people [273, 296], this is the first time that histopathological scores have been associated with reduced vitamin D concentrations.

It is unclear if the low 25(OH)D concentrations observed in inflammatory diseases are causally related to inflammation or if lower vitamin D status occur as a consequence of the inflammatory process itself. Serum 25(OH)D concentrations have been shown to decrease following elective surgical procedures in human patients, coupled with increases in inflammatory markers such as CRP [148]. This suggests that vitamin D is a negative acute phase reactant [148]. Serum 25(OH)D concentrations also fall rapidly in other inflammatory conditions such as acute pancreatitis [306]. Increases in pro-inflammatory cytokines have been proposed to be the cause of decreases in serum 25(OH)D seen in acute inflammation. For example, changes in serum 25(OH)D over several weeks corresponded with increases in serum pro-inflammatory cytokines including: TNF- α , Interferon-gamma (IFN- γ), IL-1 β , Granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-6 concentrations in patients undergoing knee arthroplasty [149]. In addition, changes in vitamin D status following inflammation may relate to changes in vitamin D binding proteins. For example, serum vitamin D binding protein decreases in the face of acute inflammation [246].

However, not all studies looking at illness events associated with acute inflammation have demonstrated decreases in serum 25(OH)D concentrations. For example, acute myocardial infarction is associated with increased markers of inflammation but serum 25(OH)D concentrations remained unaltered following infarction [307]. Studies in patients with tuberculosis have also demonstrated decreases in vitamin D are not seen during acute phase responses [308].

Hypovitaminosis D may contribute to the initiation of intestinal inflammation. Studies using experimental models of inflammatory bowel disease have demonstrated that vitamin D can have an anti-inflammatory effect. For example, in a murine model of IBD, calcitriol was shown to inhibit a number of genes involved in regulating TNF- α production and signalling [309]. Furthermore, VDR knockout mice produce significantly higher cytokine concentrations in response to chemically induced gastrointestinal inflammation than wild type mice [248]. These findings suggest that vitamin D is important in regulating gastrointestinal inflammation. However, it is not clear whether these findings can be extrapolated directly to patients with IBD. For example markers of inflammation including: CRP, erythrocyte sedimentation rate (ESR), TNF- α , IL-17, IL-10 and vascular endothelial growth factor concentrations did not change following vitamin D₃ supplementation in people with IBD [231]. Therefore, further work is needed to determine the role of vitamin D in the treatment of inflammatory bowel disease in people and dogs.

Recent investigations also demonstrate that intestinal epithelial vitamin D receptor is important for the regulation of mucosal inflammation by maintaining the integrity of the intestinal mucosal barrier [310] and VDR signalling can inhibit intestinal inflammation [260]. However, expression of the VDR by intestinal epithelial cells is down-regulated by

mucosal pro-inflammatory cytokines [262, 310]. These results suggest that inflammation interferes with the immunomodulatory effects of vitamin D.

There are a number of limitations in this study. The retrospective nature of this study made it impossible to standardise the drugs and dietary treatments dogs received prior to referral for assessment and diagnosis of their CE. These treatments may have affected the changes in inflammation markers seen and may have altered serum 25(OH)D concentrations. It would be also be desirable to have assessed other markers of local gastrointestinal inflammation in dogs such as faecal calprotectin [311]. Measurement of serum CRP concentrations may have also proved a useful assessment of systemic inflammation as CRP can detect inflammation that is unaccompanied by changes in leukocyte counts [312]. In addition, serum cytokine concentrations and gastrointestinal histology slides were not available from all dogs as some archived samples were missing.

4.5 Conclusion

In summary, this study has shown that serum 25(OH)D concentrations are inversely associated with markers of systemic inflammation in dogs and with the severity of inflammatory changes seen in histopathology samples. The results of this study indicate that clinical trials supplementing dogs with chronic enteropathies with vitamin D could provide a valuable model for assessing the response of inflammatory markers to vitamin D supplementation.

CHAPTER 5

Investigating the relationship between feline immunodeficiency virus infection and vitamin D status

5.1 Introduction

Feline immunodeficiency virus (FIV) is a retrovirus of domestic cats and other felidae which belongs to the lentivirus genus [313]. Transmission primarily occurs by horizontal spread between adult cats through bite wounds. Risk factors for infection include cats with outdoor access and cats with aggressive behaviour [314]. Once FIV infection has been acquired most cats experience a transient period of viraemia accompanied by pyrexia, lymphadenopathy, lethargy, anorexia and leukopenia [313-315]. After several weeks the viraemia subsides and cats often enter a prolonged asymptomatic phase of infection lasting many years. During this clinically asymptomatic phase there is a progressive decline in CD4⁺ T-lymphocytes [316]. Some infected cats will remain asymptomatic despite this decline in CD4⁺ T-cells; however, in other cats, the decline in CD4⁺ T-cells and resulting immune dysfunction leads to an increased susceptibility to infections, immune-mediated diseases and neoplasia [314].

Feline immunodeficiency virus shares many morphological and pathophysiological features with the human lentivirus, human immunodeficiency virus (HIV). Like HIV, FIV is characterised by tropism for lymphocytes, macrophages and cells of the central nervous system [317, 318]. In the host species, infection with each virus leads to a progressive acquired immunodeficiency syndrome. The immune dysfunction seen as a result of HIV and FIV infections includes cytokine dysregulation, inappropriate activation of immune

regulatory cells and T-cell anergy and apoptosis [319]. Overall, these conditions are associated with a pro-inflammatory state and an increase in plasma cell activity with non-specific immunoglobulin production.

A large number of studies have investigated the relationship between serum 25(OH)D concentrations and the incidence, progression and morbidity associated with HIV infection. Cross sectional studies have documented a high incidence of low vitamin D status in patients infected with HIV [77, 78]. Sub-optimal vitamin D status has been associated with lower CD4+ T-cell counts, HIV progression and AIDs related morbidity and survival [82, 84, 320, 321]. One large scale study has also identified vitamin D deficiency as an independent risk factor for all-cause mortality in HIV-positive patients [322].

The mechanistic link between vitamin D deficiency and HIV progression and AIDS related morbidity and survival may be via the marked immunomodulatory properties of calcitriol. The roles of vitamin D within the immune system include; down-regulation of pro-inflammatory cytokines, suppression of T-cell activation, shifting of T-cell responses from a Th1 to Th2 type response, regulation of monocyte chemotaxis, macrophage function and dendritic cell phenotypes and regulation of the production of antimicrobial and antiviral peptides [189, 323]. Given the known immunomodulatory function of vitamin D, it is possible that a combination of HIV infection and insufficient vitamin D could be additive in promoting immune dysfunction in patients infected with HIV. Due to the increasing evidence of the immunomodulatory effects of vitamin D and the association between sub-normal vitamin D levels and increased mortality, there is growing interest in the benefit of vitamin D supplementation in HIV-positive patients [77].

Hypothesis

Given the similarities between HIV and FIV infection, the study hypothesis is that the vitamin D status of FIV infected cats would be significantly lower than the vitamin D status of healthy control cats.

Objectives

The aim of this retrospective study was to measure and compare serum 25(OH)D concentrations in:

- Cats infected with FIV,
- Healthy cats
- A general population of hospitalised ill cats

5.2 Material and Methods

Study Population

The study was undertaken using residual serum samples from cats treated at the Hospital for Small Animals, Royal Dick School for Veterinary Studies, University of Edinburgh and clinical samples submitted to Langford Veterinary Services, University of Bristol for determination of FIV status.

Healthy control cats were defined as healthy based on history, clinical examination findings, routine haematology and serum biochemistry. Cats enrolled in this group were blood sampled for the primary clinical purposes of assessment of PCV, biochemistry, FIV status and feline leukaemia virus (FeLV) status prior to blood donation or for routine FIV/FeLV

testing prior to re-homing. Feline immunodeficiency virus and FeLV status was tested using enzyme-linked immunosorbent assay (ELISA) (SNAP® FIV/FeLV Combo Test, IDEXX Corp., Portland, Maine, USA).

The hospitalised ill cats were consecutively recruited from cats admitted to the Hospital for Small Animals, Royal Dick School for Veterinary Studies, University of Edinburgh. Cats were included in this group if they had history and clinical examination findings indicating that diagnostic interventions were warranted. Signalment, history, recent drug administration, haematology results, serum biochemistry results and final diagnosis were recorded for each case. Feline immunodeficiency virus status was tested using a (SNAP® FIV/FeLV Combo Test; or CITE Combo FIV-FeLV, Agritech Systems, Portland, Maine, USA; or Speed® FIV, Virbac Animal Health, La Seyne sur Mer, France). Cats which tested positive for FIV were excluded from the hospitalised sick group.

Samples retrieved for inclusion in the FIV-infected group were obtained from residual samples submitted to Langford Veterinary Services, University of Bristol to establish FIV status. Each of the cats recruited tested positive for FIV by ELISA (PetCheck® FIV antigen ELISA, IDEXX Corp.)

Vitamin D Measurement

Following handling of blood samples for routine diagnostic procedures, serum samples were stored at -20°C until 25(OH)D levels were measured. Samples were sent frozen in batches and 25(OH)D levels measured by high-performance liquid chromatography (HPLC). Briefly, samples were extracted using acetonitrile and applied to C18 Silica Sep-paks

(Waters Associates, Milford, MA, USA). Separation of metabolites was by straight phase HPLC (Waters Associates) using a Hewlett-Packard Zorbax-Sil Column (Hichrom, Reading, UK) eluted with hexane:propan-2-ol:methanol (92:4:4). Serum concentrations of the two major vitamers of 25(OH)D, 25(OH)D₂ (ergocalciferol) and 25(OH)D₃ (cholecalciferol), were measured separately by application to a second Zorbax-Sil Column eluted with hexane:propan-2-ol (98:2) and quantified by UV absorbance at 265 nm and corrected for recovery (sensitivity 5 nmol/L, intra- and inter-assay coefficients of variation 3.0 % and 4.2 %, respectively). Vitamer concentrations were combined and results expressed as total 25(OH)D as described previously [324]. The assay laboratory is accredited to ISO 9001:2008 and ISO 13485:2003.

Statistical Analyses

Serum 25(OH)D concentrations were compared between healthy cats, hospitalised ill cats and FIV-infected cats using a Kruskal-Wallis test with post-test Dunn's multiple comparison tests. Statistical analysis was performed with the commercial software package GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered significant.

The study was approved by The Royal Dick School of Veterinary Studies Ethical Review Committee.

5.3 Results

Signalment

The 20 cats in the healthy control group comprised of 11 domestic short-haired, 2 domestic long-haired, 2 British short hairs and 2 Maine Coon cats. Eleven were neutered males, 6 were neutered females, two were entire males and one was an entire female. The ages of the cats in the healthy control group ranged from 2 to 11 years, with a median age of 5.5 years.

The 39 cats in the hospitalised ill group comprised of 26 domestic short-haired, 5 domestic long-haired, 3 Maine Coon, 2 Bengal, 1 Burmese, 1 Norwegian Forest Cat and 1 Ragdoll cats. Twenty-four cats were neutered males, 2 were male entire and 13 were neutered females. Their ages ranged between 4 months and 18 years, with a median age of 8 years.

The cats were diagnosed with a range of medical conditions including: aortic thromboembolism (1), biliary carcinoma (1) chronic kidney disease (3), congestive heart failure (2), diabetes mellitus (1), dysautonomia (1), feline infectious peritonitis (1), fibrosarcoma (1), idiopathic chylothorax (1), idiopathic feline lower urinary tract disease (3), idiopathic megaesophagus (1) hepatic disease (1), herpes virus (1), hyperthyroidism (7), inflammatory bowel disease (2), gastrointestinal lymphoma (2), renal lymphoma (1), multicentric lymphoma (1), lymphadenopathy (1), myelodysplasia (1), oral squamous cell carcinoma (1), pyothorax (3), stomatitis (1) urolithiasis (1).

There was no clinical data available for the 59 cats in the FIV-infected group.

Serum 25(OH)D concentrations

The median serum 25(OH)D concentration in the healthy cats was 44.7ng/ml (range 14.9-61.0ng/ml). For the hospitalised ill cats the median 25(OH)D concentration was 30.9ng/ml

(range 7.1-82.40ng/ml). FIV infected cats had a median 25(OH)D concentration of 32.10ng/ml (range 5.0-62.4ng/ml).

Serum 25(OH)D concentrations were significantly different between healthy control cats, hospitalised ill cats and FIV-infected cats ($p<0.05$); (figure 1). There was a significant difference in 25(OH)D concentrations between healthy cats and FIV infected cats ($p<0.05$) and between healthy cats and hospitalised sick cats ($p<0.05$). There was no difference in the serum 25(OH)D concentrations between the hospitalised ill cats and the FIV infected cats.

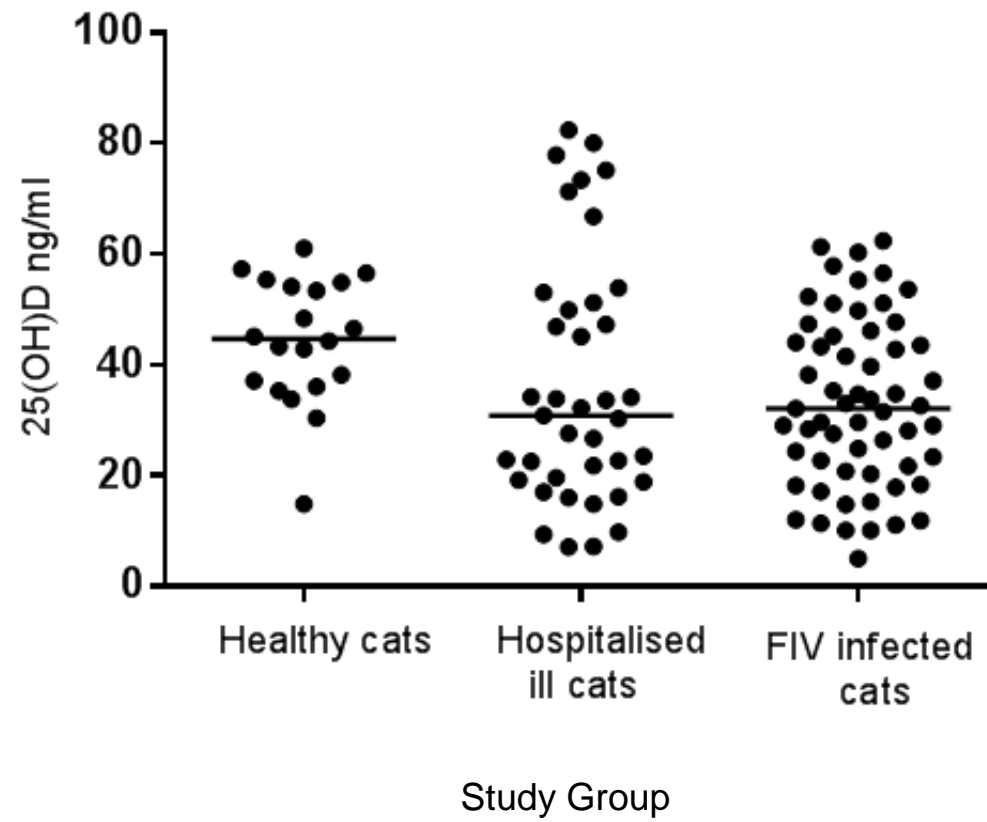


Figure 1: Serum 25(OH)D concentrations in healthy cats, hospitalised ill cats and FIV infected cats

5.4 Discussion

The central finding of this study is that FIV-infected cats have significantly lower serum vitamin D concentrations than healthy cats. This observation is similar to the findings of numerous studies in humans which have documented lower serum levels of vitamin D in HIV-positive patients [78]. Despite the well documented association, the clinical relevancy of a low vitamin D status in patients with HIV is not fully understood. Since HIV is a disease of immune dysfunction and vitamin D is known to have immunomodulatory properties, several studies have investigated whether vitamin D influences the immune response of HIV-positive patients. As an intracellular virus, HIV replicates and evades the host immune response by down regulating cell mediated immune responses including autophagy by HIV-infected macrophages [325]. Physiological doses of vitamin D have been shown to trigger autophagy and therefore inhibit HIV replication [326]. Furthermore the addition of vitamin D antagonists decreases the inhibition of HIV-1 replication [326]. In addition vitamin D pre-treatment has also been shown in vitro to decrease HIV infection, suppress viral replication, increase monocyte chemotaxis and to improve the monocyte maturation defects seen in HIV-positive patients [327]. Vitamin D has also been shown to decrease the number of *Mycobacterium avium* bacteria in macrophages from HIV-positive patients but not in HIV-negative controls, suggesting vitamin D may improve macrophage functions in HIV-positive patients [328]. Despite the significant body of evidence that has shown that vitamin D can modulate the immune response in HIV patients, it is unclear whether vitamin D supplementation can reduce morbidity and mortality in HIV-positive patients.

The second main finding of this study is that the vitamin D status of cats with FIV is not lower than FIV-negative ill cats which have been hospitalised with a range of conditions. It is unclear if low serum vitamin D concentrations are simply a non-specific change which

commonly occurs in ill cats or whether vitamin D status plays a physiological role in disease development and progression. A growing body of data in human medicine has shown that vitamin D concentrations are negatively associated with all-cause mortality and vitamin D supplementation may ameliorate morbidity [161, 216]. In chapter two this thesis also demonstrates that serum 25(OH)D are predictive of all-cause mortality in cats. The presence of vitamin D receptors on immune cells combined with experimental evidence shows that vitamin D can markedly modulate the immune system may partly explain why vitamin D status appear to have such an important association with outcome in a number of diseases.

There are several limitations in this study. Firstly, signalment and clinical data were missing from the FIV infected cohort. Although this limits the ability of this study to conclude whether reduced 25(OH)D concentrations are a direct consequence of FIV infection or due to co-morbid disease, the results do indicate that cats with FIV infections are not at any greater risk of having lower vitamin D status than FIV negative, hospitalised ill cats. The other limitation of the study is that different ELSIA tests were used to assess FIV status. In general in house tests for FIV have a similar specificities and sensitivities [329], making it unlikely that the use of different kits would adversely affect the validity of our results.

The relationship between vitamin D status and both disease susceptibility and treatment outcomes warrants further prospective investigation in a well-defined population of ill cats. In addition, future studies which investigate serum vitamin D concentrations in clinically well and clinically ill FIV-infected cats would allow the association of secondary illnesses on vitamin D status to be assessed.

5.5 Conclusion

In summary, this study has shown that FIV-infected cats have a significantly lower vitamin D status than healthy control cats. The vitamin D status of FIV-infected, hospitalised ill and healthy cats and the relationship between vitamin D status and long term prognosis in FIV-infected cats are deserving of further study.

CHAPTER 6

Thesis summary and conclusions

In summary the findings of this thesis provide further information about the role of vitamin D in companion animal diseases.

Firstly, serum 25(OH)D concentrations are predictive of short term all-cause mortality in a general hospital population of cats and also of mortality in dogs with a diagnosis of CE. This is important for a number of reasons. Prognostic markers are valuable in allowing veterinary surgeons to guide owners in decision making processes and also to highlight animals where closer monitoring or attention is beneficial. Currently it is extremely difficult to predict clinical outcomes in cats and dogs. In cats there are a very limited number of prognostic markers available, and the current methods previously reported include the Feline Acute Patient Physiologic and Laboratory Evaluation (Feline APPLE) Score [215]. This scoring system has been validated only for feline intensive care unit (ICU) patients and requires several clinical and diagnostic parameters to be assessed. A uni-variable measure such as serum 25(OH)D concentrations, applicable to a general population of ill cats, including those in ICU may provide a simpler and more readily usable predictor of mortality. A small number of prognostic markers have been reported for dogs with CEs, these include: older age, hypoalbuminaemia and higher CIBDAI scores [238]. Knowledge that vitamin D is associated with poor outcome provides further prognostic information. In addition, unlike the other prognostic markers associated with CE, serum 25(OH)D can potentially be improved with supplementation with parental or oral vitamin D analogues. Although the research in this thesis did not assess whether low serum vitamin D concentration are causally associated with poor outcomes in cats and dogs, it does highlight that randomised controlled

clinical trials with vitamin D supplementation merit consideration and may lead to potential therapeutic interventions.

The results of this thesis also highlight that hypovitaminosis D is common in ill and hospitalised cats and dogs. The results from chapter five, which compares the serum 25(OH)D concentrations of cats with FIV infections to a general population of hospitalised ill cats shows that although cats with FIV have lower 25(OH)D concentrations than healthy cats, the decrease in 25(OH)D is no greater than that seen a general population of ill cats. This is similar to the findings of a study by Lalor et al which found, that although serum 25(OH)D concentrations are lower in cats with mycobacterial infections when compared to healthy cats, they do not have lower serum 25(OH)D than a hospitalised ill control population [11]. However, in contrast other diseases may cause more profound decreases in serum 25(OH)D concentrations. For example Gow et al demonstrated that serum vitamin D concentrations were significantly lower in dogs with protein losing enteropathies compared to a hospitalised control population and dogs with CE not resulting in protein losing enteropathy [5]. Lalor et al also demonstrated that serum 25(OH)D concentrations were lower in cats with inflammatory bowel disease or gastrointestinal lymphoma than a general population of ill hospitalised cats [10]. Both Gow et al and Lalor et al demonstrated that low serum vitamin D concentrations were related to serum albumin concentrations. These results suggest some diseases have a more profound, disease specific effect on serum 25(OH)D concentrations. The effects on different disease processes on serum 25(OH)D concentrations are worthy of further study.

The results from chapter four have sought to start to investigate the association between inflammation and serum 25(OH)D in companion animals. Previously limited work has been undertaken to address this question. Investigators have examined the relationship between serum 25(OH)D concentrations and markers of inflammation by looking at CRP

concentrations in dogs with a haemoabdomen [8] and in association with the effects of strenuous exercise [293]. The results of these investigations have however been conflicting. Our results suggest that low vitamin D concentrations are associated with markers of systemic and local inflammation in dogs with a CE. Of particular interest is that a specific ‘inflammatory signature’ consisting of neutrophilia, monocytosis, and increased serum IL-2 and IL-8 concentrations were seen in these dogs. The relationship between serum 25(OH)D concentrations and these inflammatory markers, highlights areas for research to determine if low serum 25(OH)D are a cause of consequence of inflammation.

The final important finding from this work is that changes in serum 25(OH)D seen in cats and dogs are similar to those reported in ill people. This suggests that cat and dogs may provide a valuable alternative to rodent models in which the effects of vitamin D on health outcomes can be probed without the need to induce disease in otherwise healthy animals. These naturally models would be free from many of the confounding variables which make interpretation of some human studies difficult.

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APPENDICES

RESEARCH ARTICLE

Vitamin D Status Predicts 30 Day Mortality in Hospitalised Cats

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Abstract

Vitamin D insufficiency, defined as low serum concentrations of the major circulating form of vitamin D, 25 hydroxyvitamin D (25(OH)D), has been associated with the development of numerous infectious, inflammatory, and neoplastic disorders in humans. In addition, vitamin D insufficiency has been found to be predictive of mortality for many disorders. However, interpretation of human studies is difficult since vitamin D status is influenced by many factors, including diet, season, latitude, and exposure to UV radiation. In contrast, domesticated cats do not produce vitamin D cutaneously, and most cats are fed a commercial diet containing a relatively standard amount of vitamin D. Consequently, domesticated cats are an attractive model system in which to examine the relationship between serum 25(OH)D and health outcomes. The hypothesis of this study was that vitamin D status would predict short term, all-cause mortality in domesticated cats. Serum concentrations of 25(OH)D, together with a wide range of other clinical, hematological, and biochemical parameters, were measured in 99 consecutively hospitalised cats. Cats which died within 30 days of initial assessment had significantly lower serum 25(OH)D concentrations than cats which survived. In a linear regression model including 12 clinical variables, serum 25(OH)D concentration in the lower tertile was significantly predictive of mortality. The odds ratio of mortality within 30 days was 8.27 (95% confidence interval 2.54–31.52) for cats with a serum 25(OH)D concentration in the lower tertile. In conclusion, this study demonstrates that low serum 25(OH)D concentration status is an independent predictor of short term mortality in cats.

Introduction

Vitamin D is traditionally known for its role in calcium homeostasis and bone metabolism. However, it has been demonstrated that numerous types of cells express the vitamin D receptor and it is now clear that the physiological roles of vitamin D extend beyond the maintenance of skeletal health [1, 2]. Vitamin D insufficiency, which is typically assessed by measuring the

major circulating form of vitamin D, 25 hydroxyvitamin D (25(OH)D), has been associated with a number of disorders including hypertension [3], diabetes [4], cardiovascular diseases [5], cancer [6], autoimmune conditions [7] and infectious diseases [8–10]. Furthermore, low serum 25(OH)D concentrations have also been linked to all-cause mortality in the general human population [11]. Meta-analyses have demonstrated that serum 25(OH) concentrations are an important predictor of survival in people with a wide variety of illnesses [12–15].

However, interpretation of these studies is challenging. A large number of factors are known to influence serum 25(OH)D concentrations in humans including ethnicity [16–18], diet [18–20], seasonality [21], latitude and exposure to sunlight [22–24], obesity [25, 26], age [27] and gender [28]. Consequently, exploring the relationship between serum 25(OH)D concentrations and all-cause mortality in a model system in which many of these confounding factors are avoided would be of significant interest. Furthermore, a model system which did not require disease to be induced in otherwise healthy animals would allow the number of animals used in scientific research to be reduced. We predict that client owned, domesticated cats which developed spontaneous disease would be a suitable model in which to study the relationship between vitamin D and all-cause mortality. Advantages of investigating the role of 25(OH)D on health outcomes in cats include a more standard dietary intake of vitamin D since almost all cats which attend our referral veterinary hospital eat a commercial diet which is supplemented with a similar amount of vitamin D [29]. In addition, cats do not synthesize vitamin D cutaneously meaning that serum 25(OH)D concentrations are not influenced by exposure to UV radiation [30, 31].

Investigating the role of serum 25(OH)D concentrations and all-cause mortality in cats would be of interest to veterinarians since it is presently difficult to accurately predict mortality in hospitalised, ill cats. The identification of clinical measures which were predictive of mortality would be extremely helpful in providing much needed prognostic information to owners of ill cats. The aim of this study was to investigate whether serum 25(OH)D concentrations was a predictor of short term, all-cause mortality in hospitalised ill cats. We have recently demonstrated that vitamin D metabolism is altered in dogs and cats with a wide range of infectious, inflammatory and neoplastic conditions, highlighting the need to clarify the relationship between serum 25(OH)D concentrations and mortality [32–38]. We hypothesised that cats with low serum 25(OH)D concentrations would have higher mortality at 30 days post admission than cats which were vitamin D replete.

Material and Methods

Study Population

Consecutive cats examined at the Royal (Dick) School of Veterinary Studies, Hospital for Small Animals were considered eligible for inclusion in the study. Informed consent for the use of residual clinical blood samples for research purposes was obtained at admission for each cat enrolled. Ethical approval for the study was obtained from the University of Edinburgh's Veterinary Ethical Review Committee.

Clinical records were reviewed for each cat enrolled. The age, sex and breed were recorded for each cat. Survival data was obtained from clinical records or follow up telephone calls to clients and referral veterinary surgeons for each cat at day 30 post initial presentation. The following clinical information was extracted for each patient: white blood cell count, packed cell volume (PCV), serum albumin, serum creatinine, sodium concentrations, potassium concentrations, total calcium concentration and 25(OH)D concentrations. In addition, the appetite of the cats was graded as normal or reduced. Haematology variables were measured on an ADVIA(r) 2120i System with Autoslide (Siemens Medical Solutions Diagnostics Ltd

California, USA). Biochemistry parameters (serum sodium, potassium, creatinine, albumin and total calcium) concentrations were measured on an ILab650 biochemistry analyser, (Diamond Diagnostics, USA).

Following handling of blood samples for routine diagnostic procedures, serum samples were stored initially at -20°C and later moved to -70°C for longer term storage until 25(OH)D concentrations were measured as a batch. Previous studies have indicated that 25(OH)D is stable when stored at -20°C [39]. Serum concentrations of 25(OH)D₂ and 25(OH)D₃ were determined by liquid chromatography tandem mass spectrophotometry (LC-MS/MS) using an ABSciex 5500 tandem mass spectrophotometer (Warrington, UK) and the Chromsystems (Munich, Germany) 25OHD kit for LC-MS/MS following the manufacturers' instructions (intra- and inter-assay CV 3.7% and 4.8% respectively). This Supraregional Assay Service laboratory is accredited by CPA UK (CPA number 0865) and has been certified as proficient by the international Vitamin D Quality Assurance Scheme (DEQAS). Total 25(OH)D is defined as the sum of 25(OH)D₂ and 25(OH)D₃. The laboratory measuring the vitamin D metabolites were blinded to clinical data from the enrolled cats. In addition, clinicians were not aware of 25(OH)D results during the clinical management of the cats.

Statistical Analysis

Initially, we compared the 25(OH)D concentrations between cats which died within 30 days of sampling to cats which survived by a Mann-Whitney U test. In order to investigate for the presence of confounding variables we constructed a standard binary logistic regression model of death by 30 days. We included a range of clinical and biochemical data including sex, age, breed, total white blood cells, packed cell volume and serum concentrations of albumin, total calcium, creatinine, sodium and potassium. We also included an assessment of appetite as a binary variable of normal or reduced. Initially we included 25(OH)D concentrations as a linear predictor within the logistical regression model. We also categorised serum 25(OH)D concentration into 3 categories based on 33% and 66% tertiles treating the variable as a three level factor and also as low versus middle and high categories combined. We used Akaike's information criteria (AIC—a parameter penalised measure of model fit) to stepwise select variables which were to be retained to identify a final model with minimum AIC (i.e. best parameter penalised fit). P values for individual variables were calculated using Wald's test. A p-value of < 0.05 was considered to be evidence of statistical significance.

Results

Study Population

A total of 99 cats were recruited to the study. The median age of the cats was 96 months. There were 3 entire male, 56 neutered males, 1 entire female and 39 neutered female cats. Breeds included in the study were 62 Domestic Short Hairs, 8 Domestic Long Hairs, 6 Maine Coons, 5 Burmese, 3 Bengals, 2 Tomikense, 2 Siamese, 2 Ragdolls, 2 Burmese crosses, 2 Oriental Short Hair, 1 Manx Cat, 1 British Short Hair, 1 Chinchilla, 1 Burmillia and 1 Abyssinian.

There was a significant difference between the 25(OH) D concentrations between cats which were alive ($n = 80$) compared to cats which had died at 30 days ($n = 19$) ($p = 0.0022$, Fig 1). Using serum 25(OH) D concentrations as a linear predictor of survival within the logistic regression model, none of the variables, including 25(OH)D concentration, were significant predictors of mortality. Since several studies in humans have also shown a non-linear relationship between vitamin D status and mortality [11, 40, 41], we investigated whether serum 25(OH)D concentrations were a significant predictor of mortality when represented as a categorical variable. We found that cats with a 25(OH)D concentration in the lower tertile had an

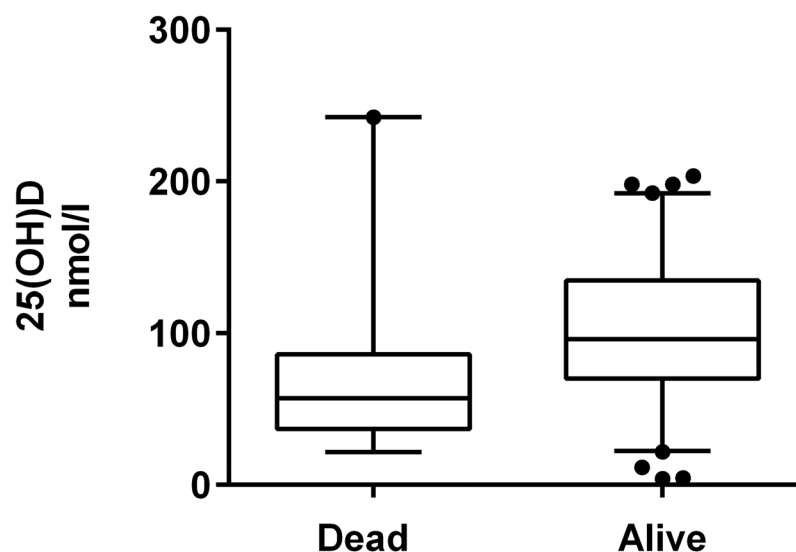


Fig 1. Box and whiskers plot of serum concentrations of 25(OH)D in nmol/l in cats which died or were alive at 30 days post admission. Box extends from 25th-75th percentiles with solid line representing the median value. Whiskers extend to 5th-95th percentiles.

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increased risk of mortality compared to cats in the middle tertile reference category (Table 1). There was no significant difference in survival between cats in the upper and middle tertile (Table 1). The only other parameters which were associated with an increased risk of mortality by 30 days were potassium concentration and a reduced appetite (Table 1).

Based on the results of the three tertile model and the epidemiological data which links low vitamin D status to poor health outcomes [12], we combined the middle and upper tertile into a single binary predictor in a third model. A serum 25(OH)D concentration in the lower tertile remained predictive of 30 day mortality (Table 2). Again, we found that potassium concentration and a reduced appetite were the only other parameters included in the third model which were predictive of survival (Table 2).

Discussion

The central finding of this study demonstrates that hospitalised ill cats with low serum 25(OH)D concentrations were less likely to survive 30 days. Using a regression model which included serum 25(OH)D concentrations as a linear variable, none of the 12 clinical, biochemical and hematological parameters, including 25(OH)D concentrations, were predictive of mortality.

Table 1. Results of logistic regression model including serum 25(OH)D concentration as three tertile categorical variable.

Variable	Odds ratio (95% CI)	P value
Potassium	4.23 (1.36–14.59)	0.0153
Reduced appetite	4.05 (1.17–17.04)	0.0370
25(OH)D category low (<73.6nmol/l)	9.51 (2.25–57.07)	0.0051
25(OH)D category middle (73.6–110.05nmol/l)	Reference category	Reference category
25(OH)D category high (>110.05nmol/l)	1.31 (0.21–8.66)	0.7681

Table only shows significant variables. (AIC = 85.50)

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Table 2. Results of logistic regression model combining vitamin D as a categorical variable using 25(OH)D as a binary predictor of lower tertile versus middle and upper tertiles combined.

Variable	Odds ratio (95% CI)	P value
Potassium	4.14 (1.34–14.21)	0.0161
Reduced appetite	4.02 (1.16–16.83)	0.0379
25(OH)D category low (<73.6nmol/l)	8.27 (2.54–31.52)	0.0008

Table only shows significant predictors. (AIC = 83.58)

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However, when we performed a second analysis in which we included serum 25(OH)D concentrations as a categorical variable, we found that low vitamin D status was an independent predictor of short term mortality.

The finding that there was a relationship between low serum 25(OH)D concentrations and mortality is consistent with numerous human studies [13, 42, 43]. In addition, the observation that there was not a linear relationship between vitamin D status and survival is also consistent with studies in human patients [11, 40]. Several human studies have reported that there is minimal benefit of having high serum 25(OH)D concentrations and a number have linked high vitamin D status to negative health outcomes [40, 44].

There are a wide range of potential mechanisms by which low vitamin D status may influence health outcomes. The vitamin D receptor is expressed on many immune cell types and it is clear that vitamin D can modulate both the innate and acquired immune responses via effects on monocytes, macrophages, dendritic cells and lymphocytes [45, 46]. Vitamin D has also been shown to profoundly modulate pro-inflammatory responses [47, 48]. The pleiotropic extra-skeletal effects of vitamin D also extends to vascular function [49] and cellular proliferation and differentiation [50]. Supplementation with vitamin D has also been shown to reduce pro-inflammatory cytokines in patients with cardiovascular disease [51]. The renin-angiotensin system is also negatively regulated by vitamin D [52, 53]. Up-regulated renin-angiotensin activity is associated with systemic hypertension, renal dysfunction, vascular damage [54] and cardiac hypertrophy [55]. Vitamin D is also inversely associated with parathyroid hormone, although this change is not seen in all patients with hypovitaminosis D [56], and excess parathyroid hormone has been related to increased risk of heart failure [57]. All of these diverse effects of low vitamin D concentrations may impact on survival in domesticated cats.

Our study also demonstrated that reduced appetite was an independent predictor of short term mortality in cats. This finding is similar to human studies where reduced appetite has been linked to poor health outcomes in elderly patients [58]. However, serum 25(OH)D concentrations remained a significant predictor of mortality when the results were corrected for reduced appetite. This suggests that the association between low serum vitamin D concentrations and mortality is not simply due to reduced dietary intake of vitamin D in hospitalised cats.

The study also demonstrated that potassium concentrations were linked to mortality with increasing potassium concentrations associated with poor survival outcomes. High serum potassium concentrations have been associated with mortality in critical care patients, even when increases in potassium are modest [59], and in patients with cardiac and renal disease [60]. The mechanism(s) by which hyperkalemia influences mortality are unclear. However raised potassium concentrations can result in altered neurological, cardiac and muscular function [59]. Furthermore, declining renal function is also associated with hyperkalemia [61] and hyperkalemia has been shown to be associated with serious infections and haemorrhage [59] which may in part explain its association with mortality.

In contrast to human medicine, little is known about the factors which are involved in all-cause mortality in cats. Previous studies have focused particularly on cats admitted to an intensive care unit (ICU), rather than across a wider hospital population [62, 63]. Therefore, the use of vitamin D to predict survival in a general hospital population is an important feature of this study. A previous reported predictor of feline survival is the Feline Acute Patient Physiologic and Laboratory Evaluation (Feline APPLE) Score [63]. This scoring system has been validated only for feline ICU patients and requires several clinical and diagnostic parameters to be assessed. A univariable measure such as serum 25(OH)D concentration may provide a simpler and more readily usable predictor of mortality.

It cannot be concluded that serum 25(OH)D is causally linked to mortality from our finding that low vitamin D status is an independent risk factor of 30 day mortality in hospitalized, ill cats. This would require further prospective studies, including randomized, placebo controlled supplementation studies of cats with low vitamin D status. In light of our finding that cats with 25(OH)D concentrations in the upper tertile had a similar incidence of mortality as cats in the middle tertile, future studies should focus on assessing whether correction of hypovitaminosis D improves health outcomes. This approach is supported by observations from human trials in critically ill patients [64]. Similarly, a study investigating the effects of vitamin D on cardiovascular morbidity and mortality, revealed that although supplementation improved overall survival, the effects were only significant in vitamin D deficient patients [65].

In conclusion, this study supports the hypothesis that low serum vitamin D status is predictive of 30 day mortality in hospitalised cats. The finding that low serum 25(OH)D concentrations are negatively correlated with survival supports the initiation of follow up clinical trials to examine the influence of vitamin D supplementation on disease outcome. Our study also indicates that domesticated cats with spontaneous illnesses may provide a valuable alternative to rodent models in which the effects of vitamin D on health outcomes can be probed without the need to induce disease in otherwise healthy animals.

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Author Contributions

Conceived and designed the experiments: RM IH. Performed the experiments: SK DGM NR NB JS SK EB AB DG HT RM IH JB SL. Analyzed the data: IH HT RM. Contributed reagents/materials/analysis tools: HT RM IH JB. Wrote the paper: SK DGM NR NB JS SK EB AB DG HT RM IH JB SL.

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Association of Vitamin D Status and Clinical Outcome in Dogs with a Chronic Enteropathy

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Background: Dogs with a chronic enteropathy (CE) have a lower vitamin D status, than do healthy dogs. Vitamin D status has been associated with a negative clinical outcome in humans with inflammatory bowel disease.

Objectives: To examine the relationship between serum 25 hydroxyvitamin D (25(OH)D) concentrations at diagnosis and clinical outcome in dogs with a CE.

Animals: Forty-one dogs diagnosed with CE admitted to the Royal Dick School of Veterinary Studies, Hospital for Small Animals between 2007 and 2013.

Methods: Retrospective review. Serum 25(OH)D concentrations were compared between dogs which were alive at follow up or had died because of non-CE-related reasons (survivors) and dogs which died or were euthanized due to their CE (non-survivors). A binary logistic regression analysis was performed to determine significant predictors of death in dogs with CE.

Results: Serum concentrations of 25(OH)D at the time a CE was diagnosed were significantly lower in nonsurvivors ($n = 15$) (median nonsurvivors 4.36 ng/mL, interquartile range 1.6–17.0 ng/mL), median survivors ($n = 26$) (24.9 ng/mL interquartile range 15.63–39.45 ng/mL, $P < .001$). Serum 25(OH)D concentration was a significant predictor of death in dogs with CE (odds ratio 1.08 [95% CI 1.02–1.18]).

Conclusions: Serum 25(OH)D concentrations at diagnosis are predictive of outcome in dogs with CE. The role of vitamin D in the initiation and outcome of chronic enteropathies in dogs is deserving of further study.

Key words: 25 (OH)D; Prognostic; Inflammatory bowel disease.

Chronic enteropathies in dogs are a major cause of morbidity and mortality.¹ A diagnosis of chronic enteropathy (CE) is made in dogs which have a several week history of gastrointestinal signs such as weight loss, vomiting, and diarrhea, and the absence of an underlying etiology based on diagnostic evaluation and the presence of an inflammatory infiltrate within gastrointestinal biopsies.² The pathogenesis of CE in dogs is considered to be multifactorial and includes factors such as abnormal mucosal immunity, disrupted epithe-

Abbreviations:

25(OH)D	25 hydroxyvitamin D
AIC	akaike information criteria
ALT	alanine transaminase
ALP	alkaline phosphatase
IBD	inflammatory bowel disease
CE	chronic enteropathy
CPA	Clinical Pathology Accreditation
CIBDAI	canine inflammatory bowel disease activity index
HPLC	high performance liquid chromatography
(TNF)- α	tumor necrosis factor alpha
VDR	vitamin D receptor
WSAVA	World Small Animal Veterinary Association

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Dogs were recruited from, and investigated at, the Hospital for Small Animals at the Royal (Dick) School of Veterinary Studies, The University of Edinburgh. Serum 25 hydroxyvitamin D measurement from cases recruited at the Hospital for Small Animals was performed at the Vitamin D research laboratory at the University of Manchester.

Previous presentations: These results have not been presented at any meetings.

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lial barrier function, altered intestinal microbial flora, environment, and genetics.^{2,3}

Dogs with CE have lower serum concentrations of 25 hydroxyvitamin D (25(OH)D), which is the vitamin D metabolite widely used to assess vitamin D status, than do healthy dogs and hospitalized dogs with nongastrointestinal illnesses.^{4,5} In addition, the severity of the clinical signs, as assessed by the canine inflammatory bowel activity index (CIBDAI), correlates with serum 25(OH)D concentrations in dogs with a CE.⁵ However, the prognostic significance of serum 25(OH)D concentrations has not been investigated in dogs with a CE.

In contrast, the relationship between vitamin D status and IBD has been extensively explored in human medicine. In human patients with IBD, notably ulcerative colitis and Crohn's disease, vitamin D deficiency is a frequent finding.^{6–9} Higher predicted vitamin D status has been associated with a reduced risk of developing Crohn's disease.¹⁰ Among people with IBD, 25(OH)D concentrations are related to disease severity scores and

patient quality of life.^{11–13} Low plasma 25(OH)D concentrations have also been associated with an increased risk of surgery and longer hospitalization periods in patients with either Crohn's disease or ulcerative colitis.¹⁴ In addition, normalization of 25(OH)D concentrations in patients with Crohn's disease has been associated with a reduction in the risks associated with surgery.¹⁴ Vitamin D status has also been linked to IBD treatment outcomes, as low pretreatment vitamin D status has been associated with reduced durability of response to anti-tumor necrosis factor (TNF)- α treatment.¹⁵ Studies have also demonstrated improvements in disease activity scores and quality of life scores in Crohn's disease patients supplemented with oral vitamin D.^{16,17}

The hypothesis of this study was that the vitamin D status would not be different in dogs that died or were euthanized because of the complications associated with their CE compared to dogs which were alive at follow up or had died because of diseases unrelated to their CE. The aim of this study was to measure serum concentrations of 25(OH)D, alongside a range of clinical and biochemical variables, in dogs with a confirmed diagnosis of CE and known clinical outcome.

Material and Methods

Study Population

The records of dogs referred to the Hospital for Small Animals, Royal Dick School of Veterinary Studies for investigation of chronic gastrointestinal disease (more than 3 weeks in duration) between 2007 and 2013 were retrospectively reviewed. Dogs were considered eligible for inclusion in the study if they had presenting clinical signs consistent with a CE, which included any of the following: vomiting, diarrhea, increased borborygmi, abdominal pain, increased or decreased appetite, and weight loss. All dogs were considered eligible if they had histopathological evidence of inflammation within the small or large intestine and there were no clinically relevant abnormalities detected on hematology, biochemistry, or abdominal ultrasonography, which were not attributable to CE. In addition, a stored frozen serum sample from each dog, collected at the time of diagnosis was required for retrospective analysis of 25(OH)D concentrations. Hematology variables (total white blood cell count, mature neutrophils, band neutrophils lymphocytes, monocytes, eosinophils, basophils, total red blood cell counts, packed cell volume, hemoglobin, mean cell volume, mean cell hemoglobin concentration, and platelet number) were measured on ADVIA(r) 2120i System with Autoslide.^a Biochemistry variables (albumin, alanine transaminase [ALT], alkaline phosphatase [ALP], bile acids, bilirubin, total calcium, creatinine, globulin, phosphate, potassium, sodium, chloride, urea, and glucose) were measured on an ILab650 biochemistry analyzer.^b Clinical records were also reviewed for the results of fecal parasitology. Canine inflammatory bowel disease activity index (CIBDAI) scores were calculated as follows: appetite, activity levels, vomiting, fecal consistency, fecal frequency, and weight loss were each scored from 0 to 3. A score of 0 indicated no changes were present, a score of one indicated mild changes, 2 moderate changes, and 3 severe changes. In addition, a score of one point was added if the feces contained blood or mucus. The area of intestinal tract which was biopsied (duodenum or duodenum and colon) was determined based on the presenting clinical signs and was at the discretion of the primary clinician managing the case. A diagnosis of CE was made if there was histological evidence of intestinal inflammation and no clear underlying cause.

Vitamin D Analysis

Serum samples retained for 25(OH)D measurement were frozen after being used for routine biochemical analysis. They were stored at -70°C before being sent to the laboratory for analysis on dry ice. Serum 25(OH)D has been shown to be stable under these conditions.¹⁸ Serum concentrations of 25(OH)D were measured as previously described in detail.^{19,20} Samples were extracted using acetonitrile and applied to C18 Silica Sep-paks. Separation of metabolites was by straight phase high performance liquid chromatography (HPLC)^c using a Hewlett-Packard Zorbax-Sil Column^d eluted with hexane:propan-2-ol:methanol (92 : 4 : 4). Serum 25(OH)D₂ and 25(OH)D₃ were measured separately by application to a second Zorbax-Sil Column eluted with hexane:propan-2-ol (98 : 2) and quantified by ultraviolet absorbance at 265 nm and corrected for recovery (sensitivity 5 nmol/l, intra- and interassay coefficients of variation 3.0 and 4.2%, respectively).²¹ Total 25(OH)D was defined as the sum of 25(OH)D₂ and 25(OH)D₃. This laboratory is accredited by CPA UK (CPA number 0865) and has been certified as proficient by the international Vitamin D Quality Assurance Scheme (DEQAS).

Histopathology

Where available, the slides of the original duodenal biopsies were reviewed by a single veterinary pathologist. A qualitative scoring system (World Small Animal Veterinary Association [WSAVA] Standards for the Diagnosis of Gastrointestinal Inflammation in Endoscopic Biopsy Samples)²² was used to assess the degree of inflammation. The template for this system assesses the following histological changes (villous stunting, epithelial injury, crypt distension, lacteal dilatation, and mucosal fibrosis) and inflammatory infiltrates (intraepithelial lymphocytes, lamina propria lymphocytes and plasma cells, lamina propria eosinophils, and lamina propria neutrophils). The changes for each of the variables listed were graded as normal (0), mild (1), moderate (2), or severe (3). The sums of all these variables were added together to determine an intestine inflammatory score which ranged from 0 (normal) to 30 (very severe).

Outcome

For each dog enrolled, follow-up data was obtained by reviewing clinical records and by telephone contact with referring veterinary surgeons and owners. Outcome was recorded as survivors if the dogs were alive at follow up or had died because of a non-CE-related illnesses or nonsurvivors if dogs had died or were euthanized because of the complications associated with CE.

Statistical Analysis

Univariable measures were compared between dogs which survived and nonsurviving dogs using a Mann–Whitney *U*-test. A Fisher's exact test was used to compare the sex of surviving and nonsurviving dogs. A binary logistic regression model was used to estimate the association between outcome (survivors versus nonsurvivors) and serum 25(OH)D concentrations conditional on a range of other candidate predictors. Stepwise selection of variables was used to minimize Akaike Information Criteria (AIC), which is a parameter-penalized measure of model fit. Duodenal histology scores were classified into 3 approximate tertiles (low <7, medium 7–8, high >8) for the regression model. The statistical analysis was performed using R statistical system (R Development Core Team 2012).

Ethical Review

Informed consent for the use of residual clinical blood samples for research purposes was obtained at admission for each dog enrolled. Ethical approval for the study was obtained from the University of Edinburgh's Veterinary Ethical Review Committee.

Results

Signalment

Forty-one dogs were included in the study. There were 15 nonsurvivors and 26 survivors. In the nonsurvivors group, 2 dogs were intact males, 7 were neutered males, 1 was an intact female, and 5 were neutered females. In the survivors group, 7 dogs were intact males, 11 were neutered males, and 8 were neutered females. Breeds in the nonsurvivors groups included Border Collie (n = 1), Boxer (n = 3), Cavalier King Charles Spaniel (n = 1), Cross Breed (n = 3), German Short Haired Pointer (n = 1), Hungarian Vizsla (n = 1), Italian Greyhound (n = 1), Pyrenees Mountain Dog (n = 1), Springer Spaniel (n = 1), Staffordshire Bull Terrier (n = 1), and West Highland White Terrier (n = 1). In the survivors group, breeds included Border Terrier (n = 1), Boxer (n = 7), Cocker Spaniel (n = 1), Cavalier King Charles Spaniel (n = 1), Chinese Crested (n = 1), Cross Breed (n = 1), Irish Setter (n = 2), Labrador Retriever (n = 2), Lurcher (n = 2), Rottweiler (n = 1), Shar-pei (n = 1), Shetland Sheep Dog (n = 1), Springer Spaniel (n = 1), Staffordshire Bull Terrier (n = 1), Toy Poodle (n = 2), and Yorkshire Terrier (n = 1).

Clinical Findings

The median duration of clinical signs at diagnosis was 3 months in the nonsurvivors (range 1–10 months) and 3.5 months (range 0.75–24 months) in the survivors group. Hematology, biochemistry, and abdominal ultrasonography findings did not reveal any clinically relevant abnormalities in any of the 41 dogs which could not be attributed to their CE. Fecal parasitology was performed in 36 dogs and did not reveal evidence of parasitic infection in any of the samples. Twelve dogs underwent gastroduodenoscopy and 29 dogs had both gastroduodenoscopy and colonoscopy.

Histopathological examination of duodenal biopsies in the nonsurvivors revealed lymphoplasmacytic enteritis (5) and mixed lymphoplasmacytic and eosinophilic enteritis (10). In the survivor group histopathological diagnosis based on duodenal biopsies included lymphoplasmacytic enteritis (8) and mixed lymphoplasmacytic and eosinophilic enteritis (18). Follow up for the survivor group ranged from 18 to 75 months (median 27 months). In the nonsurvivors group the follow up ranged from 4 days to 24 months (median 2 months).

Outcome

Fifteen dogs died or were euthanized as a result of CE (Tables 1 and 2). The age of the dogs at presentation which subsequently died or were euthanized

because of their CE ranged from 9 to 114 months (median 96 months). Dogs which subsequently died had been treated with dietary changes and antibiotics (n = 2), dietary changes, prednisolone and antibiotics (n = 7), and dietary changes, antibiotics, prednisolone and other immunosuppressive medications (n = 6). Twenty-six dogs did not die as a result of gastrointestinal disease (Tables 1 and 2). Sixteen were alive at follow up and 10 had died because of nongastrointestinal diseases. The age of the dogs in the survivors group ranged from 6 to 96 months (median 60.5 months). Dogs in this group were treated with dietary changes and antibiotics (n = 10), dietary changes and prednisolone (n = 1), dietary changes, prednisolone and antibiotics (n = 3) and dietary changes, prednisolone, antibiotics and other immunosuppressive drugs (n = 1), diet alone (n = 10), and gastroprotectants (n = 1).

Univariable analysis revealed that serum 25(OH)D, albumin and total calcium concentrations were significantly lower in nonsurvivors compared to survivors (Table 1, Fig 1). Age and CIBDAI scores were significantly higher in nonsurvivors compared to survivors (Table 1). There was no significant difference in the number of male and female dogs between the 2 groups (Table 1).

A binary logistic regression model was performed to estimate the association between outcome and serum 25(OH)D concentrations conditional on a range of other candidate predictors. Canine inflammatory bowel disease activity index was not included in this model as it has previously been shown that this measure strongly

Table 1. Univariable analysis of clinical and biochemical variables in surviving and nonsurviving dogs. The data show the median value for each variable and the interquartile range.

	Survivors (n = 26)	Nonsurvivors (n = 15)	P Value
Age (months)	60.5 (36–73)	96.0 (60–108)	.004
Calcium mg/dL	9.68 (9.04–10.12)	8.24 (6.76–9.04)	.002
Calcium mmol/L	2.42 (2.26–2.53)	2.06 (1.69–2.26)	
Albumin mg/dL	3.34 (2.73–3.52)	2.45 (1.3–2.69)	.0007
Albumin g/L	33.35 (27.3–35.2)	24.50 (13–26.9)	
CIBDAI	6 (5–9)	10 (7–12)	.0022
25(OH)D ng/mL	24.90 (15.63–39.45)	4.3 (1.6–17.0)	.0003
Male/female	18/8	9/6	.73

Table 2. Impact on model akaike information criteria (AIC) after addition of dropped predictors. An increase in AIC represents a poorer parameter-penalized model fit.

Added Predictor	AIC
Final model which includes 25(OH)D and age	36.97
Albumin	38.97
Calcium	38.81
Sex	38.60
Histology tertile	40.31

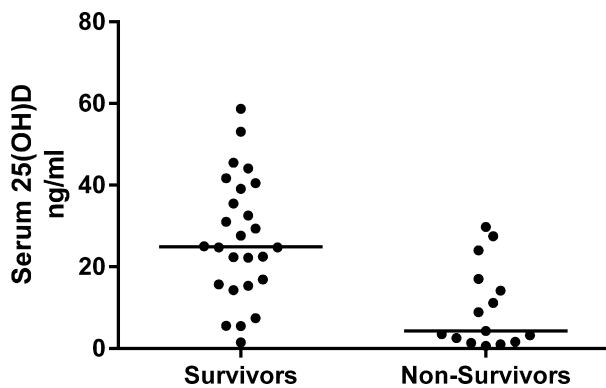


Fig 1. Serum 25 hydroxyvitamin D concentrations in surviving and nonsurviving dogs with a chronic enteropathy.

correlates with serum 25(OH)D concentrations.⁵ In this analysis duodenal histology severity scores were also included which were available for 34 of the 41 dogs. There was no significant difference in the histopathology scores between dogs which died compared to the ones which survived ($P = .06$). There were 10 dogs with a score of <7, 10 dogs with a score of 7 or 8, and 14 dogs with a score of 9 or greater. The initial model included age, sex, histopathology score as a tertile, serum calcium, albumin, and 25(OH)D concentrations. After stepwise AIC selection, the optimal predictive final model used serum 25(OH)D concentrations and age demonstrating that vitamin D status was an independent predictor of mortality in dogs with CE. The odds ratio for death was 1.08 (95% confidence interval 1.02–1.18) for vitamin D status and for age was 0.97 (95% confidence 0.93–1.00). Table 2 shows the consequences on model fit following the reintroduction of discarded predictors, demonstrating that the model based on 25(OH)D concentrations and age was optimal.

Discussion

The main finding of this study is that serum 25(OH)D concentrations are significantly lower at the time of diagnosis in dogs which died or were euthanized as a result of a CE. This is an important finding as it is presently difficult to predict outcomes in dogs with a CE. Older age, hypoalbuminemia, and higher CIBDAI scores are predictive of a poorer outcome in dogs with a CE, findings which are further supported by our research.^{1,23,24} This study, which demonstrates that 25(OH)D concentration at point of diagnosis is a prognostic marker in dogs with a CE, will provide additional prognostic information to owners and veterinarians managing dogs with a CE. Further work is needed to determine the optimal cut-off value for vitamin D as a prognostic marker.

The mechanism(s) underlying a hypovitaminosis D state in canine CE is unknown. Reduced dietary intake of vitamin D in dogs with a CE maybe an important cause of hypovitaminosis D especially as dogs do not cutaneously produce vitamin D.²⁵ As low vitamin D status in dogs with a CE has been associated with a

decreased appetite⁵, this may, in part, explain the reduced serum 25(OH)D concentrations that are frequently observed in dogs with a CE. However, dogs with a CE were found to have a lower 25(OH)D concentrations than hospitalized ill dogs with nongastrointestinal illnesses, many of which also have reduced appetites.⁵ Consequently, the influence of appetite on the vitamin D status in dogs with a CE remains unclear.

Circulating 25(OH)D is bound to vitamin D binding protein and albumin.²⁶ Enteric loss of albumin is regarded to be a significant problem in many dogs with a CE, notably in cases of protein losing enteropathies.²⁷ Consequently, loss of protein-bound vitamin D into the gastrointestinal tract could account for the low vitamin D status in some dogs with a CE. This may explain our earlier finding of a correlation between serum albumin concentration and vitamin D status in dogs with a CE.⁵ Similarly, albumin concentrations has also been shown to be a predictor of serum 25(OH)D concentration in humans with IBD.²⁸ Malabsorption might also contribute to low vitamin D status in human patients with Crohn's disease and this could potentially influence serum 25(OH)D concentrations in dogs with CE. However, while vitamin D absorption appears to be reduced in patients with Crohn's disease compared to healthy controls, there seems to be substantial variation in absorption of vitamin D in these patients.²⁹ Further studies are needed in both people and dogs with CE to clarify the potential role of vitamin D malabsorption in driving a hypovitaminosis D state.

Although hypovitaminosis D in CE has traditionally been considered to be a result of intestinal disease, there is growing evidence that hypovitaminosis D may contribute to the initiation of intestinal inflammation. Supporting evidence for a link between hypovitaminosis D and CE comes from rodent models which have demonstrated that vitamin D receptor knock out ($VDR^{-/-}$) mice are more susceptible to experimental forms of inflammatory bowel disease. For example, experimentally induced colitis in $VDR^{-/-}$ mice was significantly more severe compared to wild-type mice.^{30,31} Furthermore it has also been demonstrated that supplementing wild-type mice with vitamin D can decrease the severity of gastrointestinal inflammation with chemical induced colitis.³⁰ In addition, it has been shown that feeding mice a vitamin D-restricted diet can predispose to IBD.³² A recent meta-analysis of animal and human trials concluded that while many studies reported an improvement with supplementing vitamin D, there is insufficient evidence to currently recommend vitamin D treatment for human cases of IBD.³³ Therefore, further research is needed into the potential role of vitamin D in the management of these diseases.

The vitamin D receptor is expressed on many immune cell types and it is clear that vitamin D can modulate both the innate and acquired immune responses via effects on monocytes, macrophages, dendritic cells, and lymphocytes.^{34,35} Vitamin D has also been shown to profoundly modulate proinflammatory responses.^{36,37} Therefore, lack of vitamin D might drive abnormal inflammatory and immune processes.

Loss of tolerance to normally harmless bacterial and dietary antigens is hypothesized to be important for the development of canine CE.³ Vitamin D may also be important in regulating the immune response to commensal gut flora and maintaining normal bacterial populations. For example, dysbiosis was also reported in VDR^{-/-} mice and Cyp27B1^{-/-} mice³⁸ and dysbiosis may contribute to CE in dogs.³⁹

There is growing evidence linking vitamin D deficiency with disrupted intestinal mucosal barrier function. It has been proposed that altered epithelial barrier function, resulting in increased epithelial permeability, leads to increased exposure of the mucosal immune system to luminal antigens and that this may contribute to the initiation and perpetuation of chronic inflammation.⁴⁰ This hypothesis is supported by the observation that intestinal permeability is increased in people with naturally occurring inflammatory bowel disease and their unaffected relatives.⁴¹ Similar findings have been reported in dogs with naturally occurring CE where increased paracellular permeability has been demonstrated by lactulose to rhamnose absorption tests.^{42,43} In vitro active vitamin D metabolite, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], markedly enhances tight junctions by increasing tight junction protein expression.⁴⁴ The same study also demonstrated that VDR^{-/-} mice were more susceptible to mucosal injury than wild-type mice. These results suggest that vitamin D might be important in mucosal integrity and gastrointestinal barrier function which could contribute to CE.

Limitations of this study include the lack of standardization in treatment regime, which is a result of the retrospective nature of the study design.

In summary, this study demonstrates the serum vitamin D concentrations are predictive of clinical outcome in dogs with CE. Although, causality cannot be inferred from these results, the finding that low serum 25(OH)D concentrations are negatively correlated with outcome highlights the need to further examine the relationship between vitamin D homeostasis and disease development and outcome in dogs with CE.

Footnotes

^a Siemens Medical Solutions Diagnostics Ltd, Los Angeles, CA

^b Diamond Diagnostics, Los Angeles, CA

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^d Hichrom, Reading, UK

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RESEARCH ARTICLE

Low Vitamin D Status Is Associated with Systemic and Gastrointestinal Inflammation in Dogs with a Chronic Enteropathy

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Abstract

Introduction

Vitamin D deficiency, as assessed by serum concentrations of 25 hydroxyvitamin D (25 (OH)D), has been linked to the development of over-zealous and inappropriate inflammation in humans. However, the relationship between vitamin D status and inflammation in dogs is ill-defined. Chronic enteropathies (CE) are frequently diagnosed in client owned dogs, have a wide range of serum 25(OH)D concentrations, and represent a spontaneous model in which to probe the relationship between vitamin D and inflammation. The hypothesis of this study was that vitamin D status would be negatively associated with systemic and gastrointestinal inflammation in dogs with a CE. The aim of this study was to examine the relationship between serum 25(OH)D concentrations and markers of systemic and gastrointestinal inflammation in a cohort of dogs with CE.

Methods and Materials

Serum 25(OH)D concentrations, together with neutrophil, monocyte, eosinophil and lymphocyte counts, duodenal histopathology scores, serum IL-2, IL-6, IL-8 and TNF α concentrations and were measured in 39 dogs with histologically confirmed CE. A linear regression model examined the relationship between serum 25(OH)D status and measures of inflammation.

Results

Serum 25(OH)D concentrations were negatively associated with neutrophil and monocyte counts, duodenal histopathology scores and serum IL-2 and IL-8 concentrations. Dogs with low serum 25(OH)D concentrations typically had an inflammatory signature characterised by high monocyte and neutrophil numbers together with low lymphocyte numbers. There is

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a need to establish whether low vitamin D status is a cause or consequence of inflammation.

Introduction

The traditional functions of vitamin D relate to its role in the maintenance of calcium homeostasis and bone metabolism. In recent decades, many cell types have been shown to express the vitamin D receptor, and the physiological roles of vitamin D have been shown to extend beyond skeletal metabolism [1, 2]. Consequently, numerous studies have examined the relationship between the immune response and vitamin D status as assessed by serum 25 hydroxy-vitamin D (25(OH)D) concentrations in human patients. There is a growing body of evidence that vitamin D status is negatively associated with markers of inflammation, including circulating pro-inflammatory cytokines and acute phase proteins in a number of diseases including obesity [3, 4], inflammatory polyarthritis [5], diabetes mellitus [6], autoimmune diseases [7], inflammatory bowel disease [8, 9], and human immunodeficiency virus [10]. Furthermore, low vitamin D status has been associated with increased markers of inflammation in healthy humans [11–13].

The reasons why vitamin D status is negatively associated with inflammation is unclear. The vitamin D receptor (VDR) is found on most immune cells including macrophages, dendritic cells, T-lymphocytes and B-lymphocytes [14]. Vitamin D can promote immune tolerance by increasing T-regulatory cell populations [15], inhibiting the production of pro-inflammatory cytokines and increasing the production of anti-inflammatory cytokines [16–22]. Vitamin D is also known to enhance the innate immune response to bacteria, by increasing the production of anti-microbial peptides such as cathelicidin [18, 23].

The relationship between vitamin D status and inflammation is poorly understood in dogs. Serum 25(OH)D concentrations are commonly reduced in a number of inflammatory diseases in dogs including congestive heart failure [24], *Spirocerca lupi* infections [25], protein losing enteropathy [26, 27] and renal disease [28]. Low vitamin D status has been negatively associated with C-reactive protein (CRP) in dogs with haemoabdomen [29]. However, other studies have reported a positive association between vitamin D and CRP concentrations in racing sled dogs [30]. Consequently, further work is needed to clarify the relationship between vitamin D and inflammation in dogs.

Dogs with chronic enteropathies (CE) represent a spontaneous model in which to explore the relationship between vitamin D status and inflammation. We have previously demonstrated the dogs with CE can have a range of vitamin D concentrations, ranging from profound deficiency to sufficiency [26, 31]. However, the relationship between 25(OH)D concentrations and inflammation in dogs with CE is poorly understood. A number of markers of systemic inflammation, including serum cytokines and leukocyte profiles can be measured in dogs [32]. In addition, the WSAVA gastrointestinal histopathology scoring system for endoscopic biopsies [33] provides a standardized means of assessing the extent of inflammation within gastrointestinal biopsies from dogs with a CE.

The hypothesis of this study was that vitamin D status would be negatively associated with local and systemic markers of inflammation in dogs with a CE. The objectives were to investigate the relationship between serum concentrations of 25(OH)D and blood neutrophil, lymphocyte, monocyte and eosinophil numbers, duodenal histopathology inflammation scores, and serum cytokine concentrations in dogs with histologically confirmed CE.

Material and Methods

The records of dogs referred to the Hospital for Small Animals, Royal (Dick) School of Veterinary Studies for investigation of chronic gastrointestinal disease of more than three weeks in duration were retrospectively reviewed. Inclusion criteria including the following clinical signs, common reported in dogs with CE: vomiting, diarrhoea, increased borborygmi, abdominal pain, increased or decreased appetite and weight loss and also that the dogs had histopathological evidence of inflammation within the small or large intestine. Furthermore, there were no clinically significant abnormalities detected on hematology, biochemistry or abdominal ultrasonography indicative of non-gastrointestinal diseases for any of the dogs enrolled. In addition, the faeces of all dogs were negative for both helminth and *Giardia* infections.

Haematology variables, which included total white blood cell count, mature neutrophils, band neutrophils, lymphocytes, monocytes, eosinophils, basophils, total red blood cell counts, packed cell volume, haemoglobin, mean cell volume, mean cell haemoglobin concentration and platelet number were measured on ADVIA(r) 2120i System with Autoslide (Siemens Medical Solutions Diagnostics Ltd California, USA). At least a 100-white blood cell manual differential count was undertaken to establish the concentrations of neutrophils, monocytes, lymphocytes, eosinophils and basophils. Blood smears were evaluated under the direct supervision of a Board-certified veterinary clinical pathologist in every case. Biochemistry variables were measured on an ILab650 biochemistry analyser, (Diamond Diagnostics, USA). The area of intestinal tract which was biopsied (duodenum or duodenum and colon) was at the discretion of the primary clinician managing the case and based on presenting clinical signs. A diagnosis of CE was made if there was histological evidence of intestinal inflammation and no underlying aetiology was identified by on abdominal ultrasound, faecal samples or blood tests.

Serum samples retained for 25(OH)D measurement were frozen after being used for routine biochemical analysis. They were stored at -70°C before being sent to the laboratory for analysis on dry ice. Serum concentrations of 25(OH)D were measured as previously described in detail [34, 35]. Samples were extracted using acetonitrile and applied to C18 Silica Sep-paks. Separation of metabolites was by straight phase high performance liquid chromatography (HPLC) (Waters Associates, Milford, MA, USA) using a Hewlett-Packard Zorbax-Sil Column (Hichrom, Reading, UK) eluted with hexane:propan-2-ol:methanol (92:4:4). Serum 25(OH)D₂ and 25(OH)D₃ were measured separately by application to a second Zorbax-Sil Column eluted with hexane:propan-2-ol (98:2) and quantified by ultraviolet absorbance at 265 nm and corrected for recovery (sensitivity 5 nmol/L, intra- and inter-assay coefficients of variation 3.0% and 4.2%, respectively) [36]. This Supraregional Assay Service laboratory is accredited by CPA UK (CPA number 0865) and has been certified as proficient by the international Vitamin D Quality Assurance Scheme (DEQAS). Total 25(OH)D was defined as the sum of 25(OH)D₂ and 25(OH)D₃.

Canine pro-inflammatory cytokines (IL-2, IL-6, IL-8 and TNF- α) were measured from serum samples using a multiplex electrochemiluminescence immunoassay system (Meso Scale Discovery; MSD) as previously described [32]. Assay diluent (25 μL) was added to all wells, plates sealed and incubated for 30 min at room temperature on an orbital shaker (600 rpm). Samples and standards, diluted in assay diluent, were added at 25 μL per well. Plates were again sealed and incubated for a further 2 hours at room temperature with shaking. At the end of the incubation period, wells were washed three times with 200 μL phosphate-buffered saline (PBS), supplemented with 0.05% Tween 20 (Sigma-Aldrich) for 30 s, then discarded. Detection antibody was added at 25 μL per well, plates sealed and incubated for a further 1 h at room temperature with shaking. Plates were washed three times and 150 μL of MSD Read Buffer added to

each well, then electrochemiluminescence measured using the MSD Sector Imager 2400 plate reader.

A single veterinary pathologist (AP) reviewed the histopathological samples. The degree of inflammation present was assessed using a qualitative scoring system (WSAVA Standards for the Diagnosis of Gastrointestinal Inflammation in Endoscopic Biopsy Samples) [33]. Parameters which are assessed using this system include the following: histological changes (villous stunting, epithelial injury, crypt distension, lacteal dilatation, and mucosal fibrosis) and inflammatory infiltrates (intra-epithelial lymphocytes, lamina propria lymphocytes and plasma cells, lamina propria eosinophils, and lamina propria neutrophils). Changes were graded as normal (0), mild (1), moderate (2) or severe (3). The sums of all these parameters were added together to determine an intestine inflammatory score which ranged from 0 (normal) to 30 (very severe).

In order to investigate the relationship between multiple inflammatory markers and serum 25(OH)D concentrations, individual variables were examined using histograms and parameters which were not normally distributed (Anderson-Darling test) were transformed. Log10 transformation was undertaken except for square root transformation of eosinophil numbers since some eosinophil numbers were zero. The relationship between age, sex and 25(OH)D was examined using scatter plots and linear regression model. The relationship between 25(OH)D and inflammatory parameters was investigated using linear regression models. Age and sex was included in initial models in order to examine for any confounding effects. Final models were selected by removing potential confounders if their removal did not worsen model fit as assessed by the Akaike information criterion (AIC, a penalty parameter penalised measure of model fit). As the study wanted to examine whether 25(OH)D concentrations correlated to an inflammatory signature, medoid based partitioning was used to assign haematology parameters to three clusters. Three clusters was chosen on the basis of a consensus of results from 30 cluster count algorithms [37]. These clusters were identified independently of 25(OH)D concentrations. A Kruskal Wallis test was used to determine if there was difference in 25(OH)D concentrations between the three clusters [38]. Tukey and Kramer posthoc test for pairwise comparisons were then used to identify which clusters differ. Data was collected using Excel and analysed using R statistical software system [39]. A p value of <0.05 was used to define statistical significance.

The University of Edinburgh's Veterinary Ethical Review Committee approved the study. Informed consent for the storage and subsequent use of residual clinical blood samples for research purposes was obtained at admission for each dog enrolled.

Results

Thirty-nine dogs with a diagnosis of chronic enteropathies were enrolled. Breeds enrolled included; Boxer (9), cross breed (5), Labrador (2), Lurcher (2), Springer Spaniel (2), Cavalier King Charles Spaniel (2), Staffordshire Bull Terrier (2), and one of each of the following breeds; Irish Setter, Italian Greyhound, Toy Poodle, Shar-pei, Yorkshire Terrier, Pyrenees Mountain Dog, Hungarian Vizsla, Greyhound, German Short Haired Pointer, West Highland White Terrier, Cocker Spaniel, Dogue de Bordeaux, Shetland Sheep dog, Border Collie and a Border Terrier. The age range of dogs included was 6 to 136 months (median 65 months). Haematology, biochemistry, faecal parasitology and abdominal ultrasonography did not reveal any significant clinical abnormalities in any of the 39 dogs. Sixteen dogs underwent upper gastrointestinal endoscopy and 23 dogs had both upper and lower gastrointestinal endoscopy. Thirty-two dogs had duodenal biopsy samples which were available for histological review by a veterinary pathologist who was blinded to the clinical history and vitamin D status of the dogs. Only 32

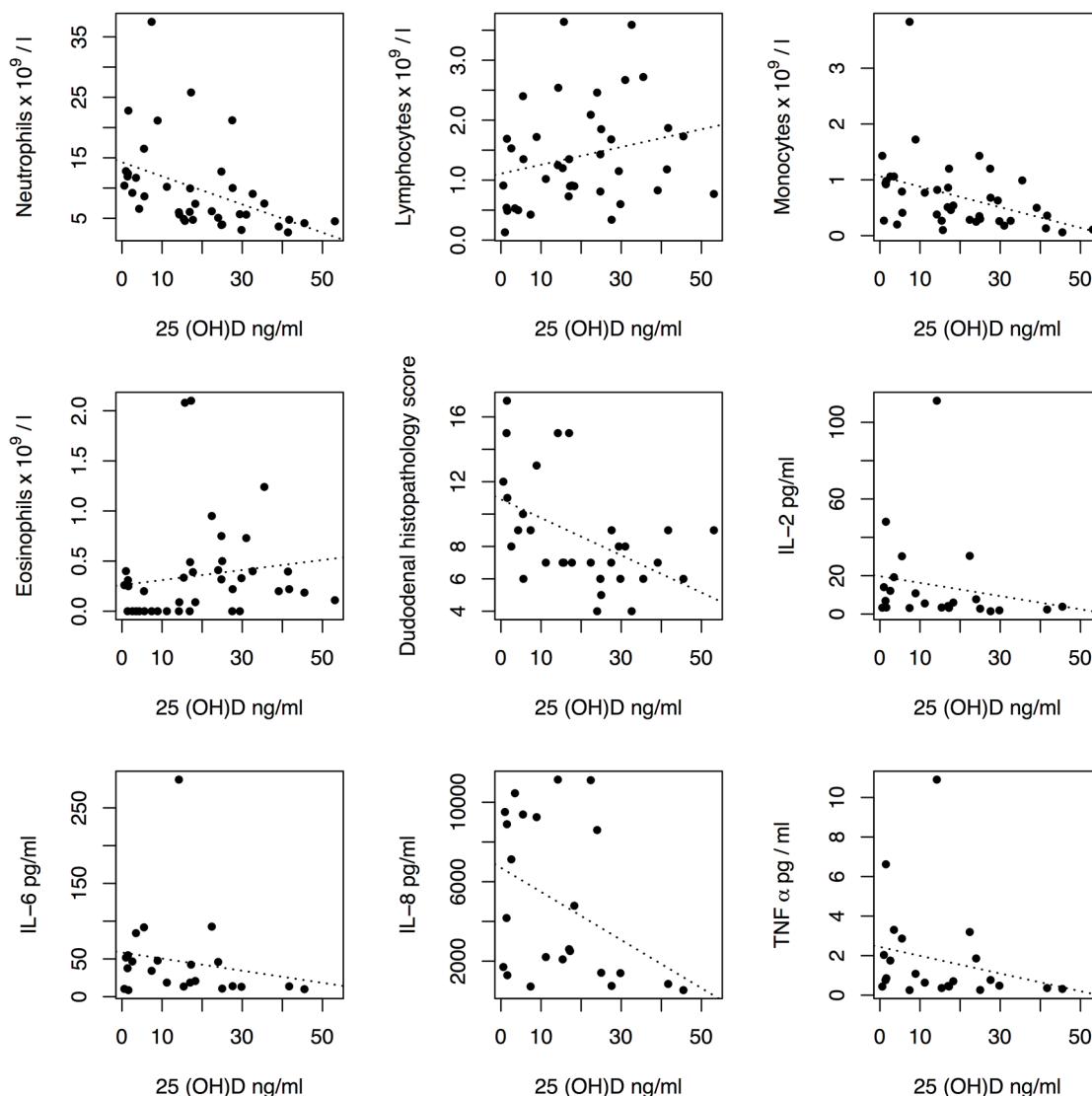


Fig 1. Relationship between haematology, serum cytokines and duodenal inflammatory score and serum 25(OH)D concentrations.

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samples were available for review as the remainder could not be retrieved from archived stores. Cytokine analysis was available in 23 cases as archived serum samples were not available for all cases.

Age and sex were not predictive of serum 25(OH)D concentrations in a linear regression model. The relationships between serum 25(OH)D concentrations and haematology, duodenal inflammation score and serum cytokine results are shown in Fig 1. Neutrophil and monocyte counts, duodenal histopathology score and serum IL-2 and IL-8 concentrations were negatively associated with 25(OH)D concentrations (Table 1). Three clusters were identified in the haematology data (Figs 2 and 3). There was a significant difference in 25(OH)D concentrations between the three clusters ($p = 0.009$). Post-test analysis revealed a significant difference between clusters 2 and 3 ($p = 0.006$).

Table 1. Results of logistic regression model of inflammatory parameters on serum 25(OH)D concentrations.

Variable	Co-efficient (SE)	P value
Neutrophil number (log10)	-0.011 (0.003)	<0.001 *
Monocyte number (log10)	-0.016 (0.004)	<0.001 *
Eosinophil number (sqrt)	0.008 (0.004)	0.09
Lymphocyte number (log10)	0.005 (0.004)	0.16
Duodenal histopathology score (log10)	-0.012 (0.004)	0.006 *
IL-2 (log10)	-0.015 (0.007)	0.048 *
IL-6 (log10)	-0.011 (0.006)	0.09
IL-8 (log10)	-0.018 (0.006)	0.01 *
TNF- α (log10)	-0.014 (0.007)	0.06

*denotes statistical significance $P < 0.05$

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Discussion

The central finding of this study is that serum 25(OH)D concentrations negatively correlate with systemic markers of inflammation including neutrophil and monocyte counts, serum IL-2 and IL-8 concentrations and local inflammation as measured by duodenal histopathology scores. Linear regression and medoid based partitioning analysis demonstrated that dogs with low vitamin D status had an inflammatory signature consisting of high neutrophil and monocyte counts and low lymphocyte numbers. A typical stress and/or inflammatory leukogram in dogs consist of neutrophilia, monocytosis and lymphopenia. These changes provide evidence of an association between reduced serum 25(OH)D concentrations and systemic inflammation in this population of dogs.

The results of this study also show that IL-2 and IL-8 concentrations are increased in the serum of dogs with CE and associated with lower 25(OH)D concentrations. Interleukin-2 acts on activated lymphocytes resulting in their expansion, and differentiation into cytotoxic lymphocytes, natural killer cells and T helper cells [40]. Interleukin-2 is under direct transcriptional regulation by 1,25(OH) $_2$ D and can decrease IL-2 production [41]. Culture of T lymphocytes with vitamin D from patients with systemic lupus erythematosus decreased production of IL-2 [42]. Similarly, vitamin D supplementation has been associated with decrease in circulating IL-2 concentrations in children with atopy [43]. In addition, increased IL-2 concentrations have been reported in vitamin D insufficient adults [44]. Serum IL-8 concentrations were also increased in this population of dogs with CE and lower 25(OH)D concentrations. In human IBD, IL-8 is an important inflammatory mediator, playing a role in the initiation and maintenance of IBD by recruiting neutrophils into the inflamed gastrointestinal tract [45]. An increase in serum IL-8 concentrations have also been reported in human patients with IBD [46]. Vitamin D has been shown to exert anti-inflammatory effects by decreasing IL-8 production in intestinal cell cultures [47]. Our results also indicate that low serum vitamin D concentrations are associated with more severe inflammation as assessed by duodenal histopathology scores. This provides evidence of an association between intestinal inflammation and reduced vitamin D concentrations.

It is unclear if the low 25(OH)D concentrations observed in inflammatory diseases are causally related to inflammation or if lower vitamin D status occur as a consequence of the inflammatory process itself. Serum 25(OH)D concentrations have been shown to decrease following elective surgical procedures in human patients, coupled with increases in inflammatory markers such as CRP, suggesting vitamin D is a negative acute phase reactant [48]. Serum 25(OH)D

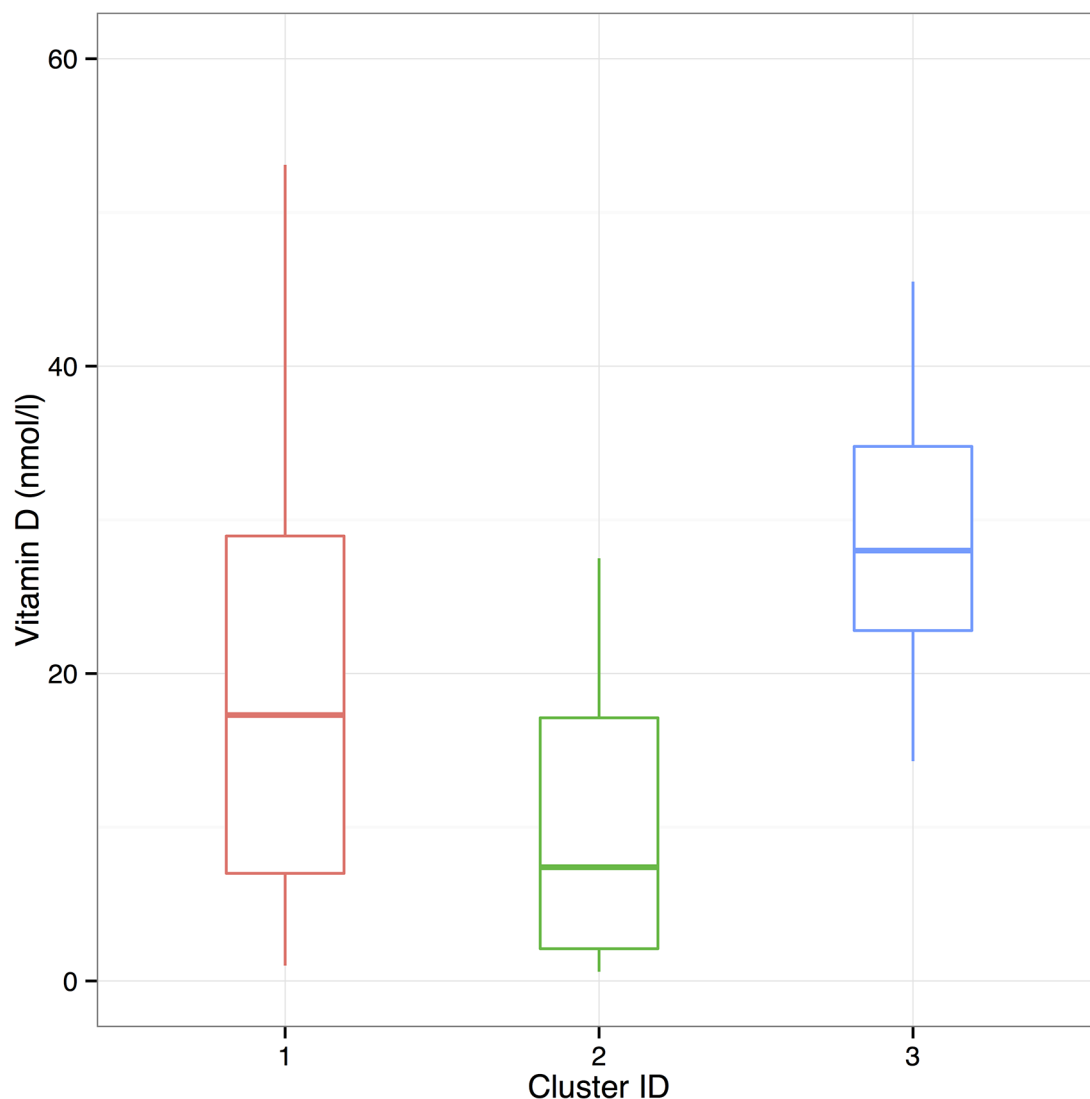


Fig 2. Box and whisker plot of serum 25(OH)D concentrations by cluster. The box extends from 25% to 75% percentile with the median and the whiskers extend to limits of the data.

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concentrations also fall rapidly in other inflammatory conditions such as acute pancreatitis [49]. Increases in pro-inflammatory cytokines have been proposed to be the cause of decreases in serum 25(OH)D in acute inflammation. For example, changes in serum 25(OH)D over several weeks corresponded with increases in serum pro-inflammatory cytokines, including TNF- α , IFN- γ , IL-1 β , GM-CSF and IL-6 concentrations, in patients undergoing knee arthroplasty [50]. However, not all studies looking at illness events associated with acute inflammation have demonstrated decreases in serum 25(OH)D concentrations [51, 52]. In addition, changes in vitamin D status following inflammation may relate to changes in vitamin D binding proteins. For example, serum vitamin D binding protein decreases in the face of acute inflammation [53].

Numerous studies have investigated the potential benefit of vitamin D supplementation in a range of inflammatory diseases. In ill patients, vitamin D administration reduces some markers of inflammation in early chronic kidney disease [54], end-stage renal disease [55], congestive

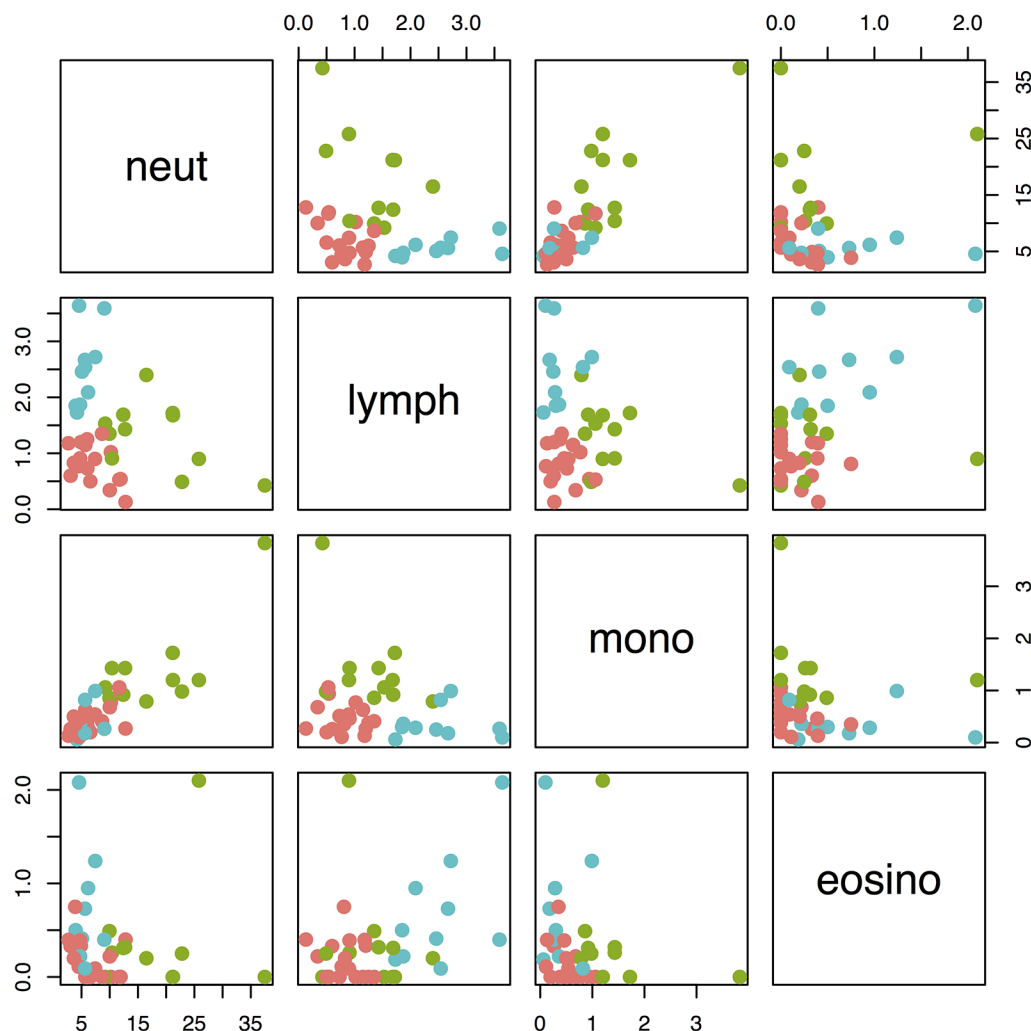


Fig 3. Pair-wise scatter plot of number of neutrophils (neut), monocytes (mono), eosinophils (eosino) and lymphocytes (lymph) labelled by cluster (red cluster 1, green cluster 2, blue cluster 3). Values on x and y axis denote cell concentrations $\times 10^9/l$.

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heart failure [56], systemic lupus [57] and colorectal adenoma [58]. In addition markers of inflammation decreased in elderly women with vitamin D insufficiency in response to supplementation with a mega-dose of vitamin D3 [59]. However, other studies investigating the effects of vitamin D supplementation on markers of inflammation have failed to demonstrate *in-vivo* anti-inflammatory effects. For example, high dose vitamin D treatment did not result in a decrease in inflammatory markers in people with low vitamin D status diagnosed with: pre-diabetes and type 1 diabetes [6, 60], hypertension [61] and urticaria [62]. Furthermore, increases in pro-inflammatory cytokines were documented in patients with osteoporosis after vitamin D supplements were administered [63]. There are many potential explanations for why the results of different studies reported differing effects of vitamin D supplementation on markers of inflammation. These include differences in the duration for which vitamin D supplementation was given, the type of vitamin D used for supplementation and the effects of various diseases. In one meta-analysis it appears that the benefits of vitamin D in reducing inflammatory markers is dependent on the disease state studied and initial, pre-treatment 25 (OH)D concentrations [64]. The benefits of vitamin D supplementation appear to be most

evident in markedly inflammatory diseases where serum 25(OH)D concentrations are initially low.

Although hypovitaminosis D in CE has traditionally been considered to be the result of intestinal disease, there is growing evidence that hypovitaminosis D may contribute to the initiation of intestinal inflammation. Intestinal epithelial vitamin D receptor is important in the regulation of mucosal inflammation by maintaining the integrity of the mucosal barrier [65], suggesting hypovitaminosis D may also perpetuate inflammation. However, the expression of VDR is influenced by mucosal inflammation and is down-regulated by mucosal pro-inflammatory cytokines [65, 66]. Studies in experimental models of inflammatory bowel disease have demonstrated that vitamin D can have an anti-inflammatory effect. For example, in a murine model of IBD, 1,25(OH)₂D was shown to inhibit a number of genes involved in regulating TNF- α production and signaling [67]. Furthermore, VDR knockout mice produce significantly higher cytokine concentrations in response to chemically induced gastrointestinal inflammation than wild type mice [68]. These findings suggest that vitamin D is important in regulating gastrointestinal inflammation. However, it is not clear whether these findings can be extrapolated directly to patients with IBD. For example, CRP, ESR, TNF- α , IL-17, IL-10 and vascular endothelial growth factor concentrations did not change following vitamin D₃ supplementation in people with IBD [69]. Therefore, further work is needed to determine the role of vitamin D in the treatment of inflammatory bowel disease in people and dogs.

There are some limitations to this study. The retrospective nature of this study made it impossible to standardise the drugs and dietary treatments the dogs received prior to referral. In addition, serum cytokine concentrations and gastrointestinal histology slides were not available from all dogs.

Conclusion

In summary, this study has shown that serum 25(OH)D concentrations are inversely associated with markers of systemic inflammation in dogs with CE, and with severity of inflammatory changes seen in histopathology samples. Further studies are required to assess whether low vitamin D status is a cause or a consequence of inflammation.

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Author Contributions

Conceived and designed the experiments: HT AG RM IH. Performed the experiments: HT SK JC AG RM IH JB EM AP. Analyzed the data: HT RM IH AP AG. Contributed reagents/materials/analysis tools: HT SK JC AG RM IH JB EM AP. Wrote the paper: HT SK JC AG RM IH JB EM AP.

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